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REVERSED-PHASE LIQUID CHROMATOGRAPHY OF ISOMERIC ALKYLBENZENES

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(Received December 29th, 1980)

SUMMARY

The separations of isomeric C_1–C_4 alkylbenzenes and polymethylbenzenes on the reversed-phase columns SAS-Hypersil, ODS-Hypersil and C_{22}-Magnusil have been compared. The correlations between log k' and the structural parameters carbon number, molecular connectivity index and hydrophobic surface areas were determined. The retentions were also compared with octanol–water and heptane–water partition coefficients.

INTRODUCTION

Although many studies have been carried out on the separation of the environmentally important polynuclear aromatic hydrocarbons (PAHs) by both normal- and reversed-phase liquid chromatography[1], the monocyclic aromatic hydrocarbons have not been examined in detail. In early studies, normal-phase separations on alumina[2,3] and silica[4] were carried out with pentane or fluorocarbons as eluents, but it was not easy to relate the retentions to structural features. Recently, hydroxylated silica has been used, and linear relationships have been determined for the *n*-alkylbenzenes and polymethylbenzenes between log capacity factor and number of carbon atoms[5].

Individual alkylbenzenes have been examined on reversed-phase columns by a number of authors[6–10], but the only systematic study has been of the C_1–C_9 *n*-alkylbenzenes, which were found to show a linear relationship between log capacity factor (k') and carbon number[11].

The absence of polar functional groups in the alkylbenzenes suggests that they could be useful models to test the solvophobic theory of retention, which relates k' to the solute hydrocarbonaceous surface area[12,13]. However, other studies of hydrophobicity, with use of octanol–water distribution coefficients, have suggested that, for non-polar molecules, molecular volumes show a closer relationship than areas[14]. The concept of molecular connectivity index has similarly been proposed to relate structure to partition coefficients and hence to retention[15].

In the present work, the isomeric C_1–C_4 alkylbenzenes and mono- to tetramethylbenzenes have been examined on three reversed-phase columns. The k' values have been compared with the carbon numbers, reported surface areas and volumes and

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TABLE I
CAPACITY FACTORS OF ALKYLBENZENES ON REVERSED-PHASE COLUMNS AND RELATED STRUCTURAL FACTORS AND PARTITION COEFFICIENTS

Compound	*Capacity factors (solvent ratio, methanol–water)*				*Structural factors*			$\log P_{octanol-water}$ *(ref. 18)* / $\log P_{heptane-water}$ *(ref. 19)*	
	SAS-Hypersil		*ODS-Hypersil (70:30)*	C_{22}*-Magnusil (50:50)*	$\chi^{\star}$	*Surface area***	*Volume****		
	(40:60)	*(50:50)*							
Benzene	1.6	1.00	1.40	0.94	2.000	240.7	80.33	2.13	2.26
Toluene	3.3	1.67	2.60	1.76	2.410	273.9	98.81	2.69	2.85
Ethylbenzene	6.5	2.67	4.50	2.76	2.971	302.3	115.79	3.15	
o-Xylene	5.7	2.45	4.50	2.85	2.827	298.4	117.33	3.12	3.39
m-Xylene	6.5	2.67	4.96	3.15	2.821	309.1	117.33	3.20	3.54
p-Xylene	6.5	2.78	4.96	3.11	2.821	309.1	117.33	3.15	3.45
n-Propylbenzene	13.8	4.78	8.50	5.06	3.411	334.1	132.77	3.62	
Isopropylbenzene	11.1	4.00	8.10	4.23	3.354	326.0	132.77	3.66	
1,2,3-Trimethylbenzene	10.4	3.78	7.60	4.52	3.244				
1,2,4-Trimethylbenzene	11.6	4.33	8.50	5.23	3.238	331.6			
1,3,5-Trimethylbenzene	12.7	4.44	9.40	5.49	3.232		135.85	3.42	
n-Butylbenzene	–	8.89	15.7	9.47	3.971				
Isobutylbenzene	–	8.44	14.3	8.52	3.827				
sec.-Butylbenzene	–	7.33	12.7	7.47	3.892				
tert.-Butylbenzene	–	6.22	10.7	6.23	3.661		149.74	4.11	
1,2,3,4-Tetramethylbenzene	–	6.22	12.9	8.04	3.661				
1,2,3,5-Tetramethylbenzene	–	6.88	14.2	8.51	3.655				
1,2,4,5-Tetramethylbenzene	–	6.78	14.3	8.51	3.655		154.37	4.00	
p-Methylisopropylbenzene (cymene)	–	7.44	13.5	8.21	3.571				
Biphenyl	15.9	4.56	7.50	6.26	4.071			4.04	
Naphthalene[§]	6.5	2.44	4.10	3.35	3.155		122.90	3.30	

* Molecular connectivity index calculated according to ref. 15.
** Solvent cavity surface area[13].
*** Bondi volume[14].
[§] Used as internal standard.

calculated molecular connectivity indexes. Because of the possible application of liquid chromatography to the determination of Hansch distribution coefficients[16,17], the retentions were also related to octanol–water and heptane–water partition coefficients.

EXPERIMENTAL

Liquid chromatography was carried out with the use of a Pye-Unicam XPS pump with pulse damper, a Shandon syringe injector and columns (10 cm × 5 mm) and an Altex Model 153 detector at 254 nm; solvent flow-rate was 1.0 or 1.3 ml min^{-1}.

The columns were packed with 5-μm SAS-Hypersil, ODS-Hypersil (Shandon Southern, Runcorn, Great Britain) or C_{22}-Magnusil (Magnus Scientific, Sandbach, Great Britain). Solvents were HPLC-grade methanol (Fisons, Loughborough, Great Britain) and distilled water. The alkylbenzenes were laboratory grade, obtained from a number of sources, and samples were injected, by using a stop-flow technique, as dilute solutions in methanol–water (7:3). Naphthalene was used as internal standard.

RESULTS AND DISCUSSION

Retention parameters

In an early study of a range of alkylbenzenes with differing substitution patterns and various alkyl groups, Sleight reported a correlation between log capacity factor and total number of carbon atoms[7]. Close correlations have subsequently been established for *n*-alkylbezenes and other homologous series of solutes, and the general equation $\log k'_n = pn + q$ has been derived, in which p and q are characteristic constants for a particular homologous series and column and solvent combination[11]. These values can be used to express the ability of the column to separate adjacent homologues, as the column selectivity $\alpha = k'_{n+1}/k'_n = 10^p$, the value of α varying as the polarity (ε) of the solvent.

In the present work, the k' values of 19 alkyl- and polymethylbenzenes have been determined on three reversed-phase columns (Table I), the solvents being chosen to give reasonable retention times in each instance. The ODS-Hypersil column, as expected, showed a greater retention than the Short Alkyl Silica (SAS-Hypersil) column, but, unexpectedly, the C_{22}-alkyl bonded column, rather than having greater retention, was similar to the SAS column. The relative order of elution of the monocyclic compounds was effectively the same on all three columns, and, on plotting k' for the ODS and C_{22} columns, a single line was produced (correlation 0.9971), (ODS and SAS correlation, 0.9799).

As in the earlier study, there was reasonable overall correlation between log k' of the alkylbenzenes on the ODS-column and their carbon numbers (Table II). If just the homologous *n*-alkylbenzenes were considered, as expected, a much closer correlation was found for all three columns. The values of p, q and α are similar to those reported for the C_1–C_9 alkylbenzenes on LiChrosorb RP-8 and RP-18 (ref. 11) and are in agreement with the reported trend to higher values of p and α with increased eluotropic strength of the solvent.

Although not a true homologous series, polymethylbenzenes with adjacent substituents again correspond to a systematic structural change. They also showed a

TABLE II
RETENTION-PARAMETER RELATIONSHIPS BETWEEN CAPACITY FACTORS AND CARBON NUMBERS FOR ALKYLBENZENE ON REVERSE-PHASE CHROMATOGRAPHY

Compounds	*Column*	*Mobile phase ratio (methanol–water)*	*Correlation*	*p*	*q*	*α*
Alkylbenzenes	ODS Hypersil	70:30	0.9903	0.240	−1.256	1.74
n-Alkylbenzenes	C_{22} Magnusil	50:50	0.9986	0.247	−1.510	1.77
	ODS Hypersil	70:30	0.9998	0.262	−1.424	1.83
	SAS Hypersil	50:50	0.9984	0.236	−1.658	1.72
		40:60	0.9998	0.311	−1.432	2.04
	RP-8*	100:00	–	0.066	−0.925	1.16
		90:10	–	0.101	−0.586	1.26
		80:20	–	0.154	−0.309	1.43
	RP-18*	100:00	–	0.100	−0.73	1.26
		90:10	–	0.151	−0.420	1.42
		80:20	–	0.183	−0.080	1.52
Methylbenzenes**	C_{22}-Magnusil	50:50	0.9983	0.232	−1.406	1.71
	ODS-Hypersil	70:30	0.9994	0.239	−1.274	1.73
	SAS Hypersil	50:50	0.9989	0.194	−1.157	1.56

* From ref. 11.
** Benzene, toluene, 1,2-dimethylbenzene, 1,2,3-trimethylbenzene and 1,2,3,4-tetramethylbenzene.

good linear relationship between log k' and carbon number (Table II), but with a flatter slope than for the *n*-alkylbenzenes. The different slope could be due to the increasing electron density on the benzene ring because of the polyalkyl substitution. This effect should be relatively small for a non-specific absorbent, but had a marked effect in the study on hydroxylated silica. Although retention decreased with increasing chain length of monoalkylbenzenes, polymethylation resulted in an increase in retention time[5].

As with many gas chromatography columns, it was not possible to resolve the three xylenes, although the *ortho*-isomer was well separated from *meta*- and *para*-xylene.

Two bicyclic compounds (biphenyl and naphthalene) were also examined; both were eluted much earlier than would be expected from their carbon numbers. On the ODS column, naphthalene (C_{10}) would be predicted to have a k' value of 13.8 (experimental 4.1) and biphenyl (C_{12}) a value of 42 (experimental 7.5). The additional aromatic ring in each compound apparently makes it more polar than a corresponding alkyl chain.

Correlation with physical parameters

Although the relationship between k' and number of carbon atoms has a useful general predictive power for homologues, it cannot propose the relative order of elution of isomeric compounds such as the four butylbenzenes. Attempts have been made, therefore, to devise relationships that will more closely relate structure and retention. These have invariably been based on models of the nature of the interaction between the sample and the stationary phase.

The solvophobic hypothesis proposes that the retention of a solute is dependent on the decrease in repulsion between the hydrocarbonaceous surface area of the

sample and the solvent, which occurs when the sample interacts with the non-polar stationary phase[12]. Similar considerations have been used to relate the solvent cavity surface areas of hydrocarbons with their solubilities in water[13] and the molecular areas and volumes of non-polar molecules with their partition coefficients[14].

For the alkylbenzenes examined in the present study, values have been reported for the surface area[13] of 9 compounds and for the Bondi volume[14] of 11 compounds (Table I). Comparison of these values with k' on SAS- and ODS-Hypersil columns showed a good correlation (Table III), and if the homologous *n*-alkylbenzenes only were considered, the relationship was closer. From the Bondi-volume correlation, the expected k' of naphthalene can be predicted as 5.6, in closer agreement with the experimental value of 4.1 than the earlier prediction on the basis of carbon number.

However, surface areas and volumes are not easy to determine. The readily calculated molecular connectivity index χ, which is derived from the topology of the molecule, has been shown to relate closely to water solubility and partition coefficient[15], and it has been suggested that this parameter can be related to k' values in chromatography[6,16]. The calculated values for the compounds in the present study (Table I) showed a reasonable correlation with log k' (Table III), although SAS was better than ODS. Again, if the *n*-alkylbenzenes only were considered, the correlations were improved. Because of the similarity of the relative retention on ODS and C_{22} columns, separate correlations for the latter were not determined.

Close examination suggested that, despite its relationship to the topology of the molecule, the connectivity index could not predict the order of elution of the isomeric isobutyl- and *sec.*-butylbenzenes, or the large difference in retention between *tert.*-butylbenzene ($k' = 6.23$) and 1,2,3,4-tetramethylbenzene ($k' = 8.04$), each of which had the same connectivity index, χ (3.661).

By using the connectivity index of the bicyclo compounds, the expected k' values were calculated to be naphthalene 6.65 (found 4.10) and biphenyl 21.1 (found 7.50), results similar to those found from the carbon-number relationships.

Despite claims to the contrary, it therefore appears that the connectivity index is little better than carbon number in relating k' to structure for these compounds and

TABLE III
RELATIONSHIP BETWEEN LOG CAPACITY FACTORS AND PHYSICAL PARAMETERS FOR ALKYLBENZENES ON REVERSE-PHASE COLUMNS

*Physical property**	*Correlation coefficients*** (number of compounds)*			
	*SAS-Hypersil****		*ODS-Hypersil*	
	Alkylbenzenes	*n-Alkylbenzenes*	*Alkylbenzenes*	*n-Alkylbenzenes*
Solvent cavity surface area	0.9943 (9)	0.9992 (4)	0.9951 (9)	0.9994 (4)
Bondi volume	0.9931 (11)	0.9985 (4)	0.9913 (11)	0.9995 (4)
Molecular connectivity index	0.9901 (19)	0.9995 (5)	0.9756 (19)	0.9985 (5)
Parition coefficients				
Log $P_{\text{octanol - water}}$	0.9835 (11)	0.9974 (4)	0.9707 (11)	0.9980 (4)
Log $P_{\text{heptane-water}}$	0.9948 (5)	— §	0.9982 (5)	— §

* Values from table I.
** Excludes naphthalene and biphenyl.
*** Solvent: methanol–water (50:50).
§ Insufficient samples.

generally fails to correlate with the relative retentions of isomeric compounds. Its only close correlation, that *n*-alkylbenzenes, is explicable by the incremental change of index with members of a homologous series. Overall, it shows a poorer correlation than does surface area or volume with capacity factor. Similar problems were noted by Colin and Guiochon[11], who compared k' values for isomeric C_4 and C_5 alcohols with surface area and connectivity index and found an inability to predict the relative elution of isomers.

Because of the relationship between k' values and partition coefficients, it has been proposed that liquid chromatography could be used to determine Hansch partition coefficients ($\log P_{\text{octanol-water}}$), which are important in studies of drug structure–activity relationships[16–18]. For the accurate comparison of compounds with different functional groups, it has been found necessary to impregnate hydrocarbon bonded phases with octanol[16,17], but this should not be necessary for the relative comparison of compounds of similar type.

As with physical parameters, there was a good correlation between $\log P_{\text{octanol-water}}$ and $\log k'$ for the *n*-alkylbenzenes, but, for a wider group of compounds, correlation was poorer. With a limited number of examples, there was good correlation with $\log P_{\text{heptane-water}}$.

CONCLUSION

The *n*-alkylbenzenes show good correlation between $\log k'$ and carbon number and structural parameters, but, for isomeric alkylbenzenes, the correlation is much weaker and the physical parameters are often little better than carbon number in predicting k' values.

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REVERSED-PHASE LIQUID CHROMATOGRAPHY

II. DETERMINATION OF PARTITION COEFFICIENTS OF BARBITURATES AND UREA HERBICIDES*

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(First received July 2nd, 1980; revised manuscript received December 22nd, 1980)

SUMMARY

Experimental R_M values for a series of barbiturates were determined using reversed-phase high-performance liquid chromatography. Statistically significant linear relationships were found between these R_M values and Hansch's π parameters. Relationships were also obtained between R_M values and partition coefficients determined in the system diethyl ether–dimethylformamide–water (2:1:1) and partition coefficients determined using gas–liquid chromatography. Hansch's π parameters for anilines were correlated with R_M values of some substituted urea derivatives.

INTRODUCTION

A frequently used approach to the assessment of quantitative relationships between biological activity and chemical structure is Hansch's analysis [1,2]. An important part of this analysis is the determination of the partition coefficient in a *n*-octanol–water reference system and as defined[1] by

$$\pi = \log P_x - \log P_H \quad (1)$$

where π is Hansch's parameter, P_x is the partition coefficient of a substituted compound and P_H is the partition coefficient of the corresponding unsubstituted compound.

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In previous work[3] we have studied the relationship between the R_M values of phenols, as measured by different chromatographic techniques, and Hansch's parameter π. We confirmed the validity of Collander's relationship[4]

$$\log P = bR_M + a \tag{2}$$

which holds for closely related development systems where the polar phase is water. It has been suggested[5–9] that high-performance liquid chromatography (HPLC) with bonded hydrocarbon stationary phases may be suitable for determining partition coefficients, and other workers[10,11] have studied whether it is convenient to determine partition coefficients by means of gas chromatography (GC).

The aim of our study was to investigate the relationship between the partition coefficients of barbiturates and urea herbicides, determined in different separation systems, and the corresponding R_M values, as measured by reversed-phase HPLC. We have also tested the suitability of GC for the determination of the partition coefficients of barbiturates.

EXPERIMENTAL

Chemicals and equipment

Samples of barbiturates (gifts from n. p. Léčiva, Prague, Czechoslovakia) were of analytical-reagent grade. The urea herbicides were monuron, diuron (DuPont, Wilmington, DE, U.S.A.), monolinuron, linuron (Hoechst, Frankfurt/M, G.F.R.), chlorbromuron, methobromuron (Ciba, Basle, Switzerland) and methoxuron (Sandoz, Basle, Switzerland). Solvents were redistilled before use.

Chromatography was performed on a Varian LC 8500 liquid chromatograph equipped with a UV flow detector ($\lambda_{max.}$ = 254 nm). The MicroPak CH-10 column (25 cm × 2.2 mm) (E. Merck, Darmstadt, G.F.R.) was packed with silica gel having an octadecyl chemically bonded non-polar phase (particle size 10 μm). Also used were: a Pye Unicam Type GCV gas chromatograph with flame ionization detector (FID) and glass columns (200 cm × 2 mm) packed with either 3% OV-1 on Diatomite CQ (0.100–0.120 mm) or 3% OV-17 on Diatomite CQ (0.100–0.120 mm); a Packard Model 419 gas chromatograph with FID and glass column (200 cm × 3 mm) packed with 3% NPGS + 0.75% TA on Chromaton N AW DMCS (0.100–0.125 mm). Before packing, all the columns were silanized.

In liquid chromatography the samples were injected by use of a 50-μl Hamilton 705 syringe, while in gas chromatography a 1-μl SGE Type 5 BL-RD-3 syringe was employed.

Conditions

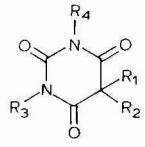
Separations on the MicroPak CH-10 column were carried out with methanol–water and dioxan–water mixtures of varying compositions as the mobile phases. The flow-rate was 60 ml/h.

In gas chromatography the temperatures of the columns, injector and of the detector were 200, 240 and 230 °C, respectively. The flow-rate of the carrier gas (nitrogen) was 40 ml/min for the 3% OV-1 packing, 55 ml/min for 3% OV-17 and 60 ml/min for 3% NPGS + 0.75% TA.

In liquid chromatography the samples were dissolved in the mobile phase to give concentrations of barbiturates of *ca.* 1 mg/ml and of urea herbicides of *ca.* 0.1 mg/ml, and 5–10 μl were immediately injected by syringe. In gas chromatography the barbiturate samples were taken from an ethanolic solution (0.1 mg/ml) and 0.5 μl were injected by syringe.

RESULTS AND DISCUSSION

The capacity factors, k', of barbiturates (1) and urea herbicides (2) were calculated according to[12]



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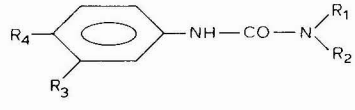

2

$$R_M = \log k' = \log (V_R - V_0)/V_0 \tag{3}$$

where V_R is the elution volume of a compound and V_0 the elution volume of an unretained compound.

We have examined the relationship between the R_M (I) and R_M (II) values of barbiturates measured in the mobile phases methanol–water (1:4) and dioxan–water (1:4) respectively, and the R_M (III) values reported by Tjaden *et al.*[13] (the values were measured on a column of a short-chain chemically bound stationary phase, *cf.*, eqns. 4 and 5). Hemetsberger *et al.*[14] showed that there are no significant differences in chromatographic behaviour between bonded short-chain *n*-alkane packings and octadecylsilyl (ODS) types of packings.

We then studied the relationship between the values R_M (I) and R_M (II) and R_M (IV) values calculated from the data of Baker *et al.*[6]; the latter were measured on a μBondapak C_{18} column (300 × 3.9 mm) using methanol–water (2:3) as the mobile phase (pH 7). The regression coefficients calculated (eqns. 6 and 7) are statistically highly significant, which shows that in the above case the R_M values measured on various commercial phases are readily comparable. Other workers[15–17] have also compared retention data measured on various types of reversed phases. On the basis of the retention data for polycyclic aromatic hydrocarbons, Ogan and Katz[15] concluded that a chromatographic analysis accomplished on one C_{18} column cannot be always duplicated on a C_{18} column from a different manufacturer. For further evaluation of this problem it would be necessary to perform comparisons of the chromatographic behaviour of different groups of compounds on various commercial reversed phases.

Next we investigated the relationship between R_M (I), R_M (II) and the logarithms of the partition coefficients, log P (I) determined in *n*-octanol–water[18,19] (eqns. 8 and 9) and log P (II) determined in diethyl ether–dimethylformamide–water (2:1:1)[20] (eqns. 10 and 11). Values of the partition coefficients are given in Table I.

The relationships obtained may be summarized as follows

Equation	*n*	*r*	*s*	*No.*
R_M (I) = 0.783 R_M (III) + 0.007	9	0.978	0.106	(4)
R_M (II) = 0.637 R_M (III) + 0.058	9	0.970	0.068	(5)
R_M (I) = 0.760 R_M (IV) + 0.451	9	0.953	0.106	(6)
R_M (II) = 0.615 R_M (IV) + 0.415	9	0.955	0.076	(7)
R_M (I) = 0.450 log P (I) + 0.165	10	0.967	0.082	(8)
R_M (II) = 0.435 log P (I) + 0.089	9	0.973	0.055	(9)
R_M (I) = 1.117 log P (II) + 0.564	8	0.895	0.160	(10)
R_M (II) = 1.095 log P (II) + 0.480	7	0.803	0.180	(11)

where n = number of compounds in the set, r = regression coefficient and s = standard deviation. All the relationships are statistically significant ($P < 0.01$). The regression coefficients calculated for eqns. 10 and 11, using the partition coefficients determined in diethyl ether–dimethylformamide–water (2:1:1), are, however, lower than those for eqns. 8 and 9 where the partition coefficients were determined in *n*-octanol–water. When evaluating these regression equations it must be remembered that the investigated compounds were partly dissociated in the mobile phase.

Reversed paper[21] or thin-layer chromatography[22–25] has also been used for measuring partition coefficients. In previous work[3] we have shown that there is a statistically significant linear relationship between the R_M values as measured by HPLC and PC or TLC.

We have also examined the relationship between the partition coefficients of

TABLE I

PARTITION COEFFICIENTS OF BARBITURATES DETERMINED BY REVERSED-PHASE LIQUID CHR MATOGRAPHY

Systems: I = MicroPak CH-10, methanol–water (1:4), flow-rate 60 ml/h; II = MicroPak CH-10, dioxan–water (1: flow-rate 60 ml/h; III = methyl silica, methanol–water (1:3), taken from Tjaden *et al.*[13].

Compound	*Substituents*			R_M *values*			*log P (I)**	*log P (II)*
	R_1	R_2	R_3	*I*	*II*	*III*		
Amobarbital	C_2H_5	$CH_2CH(CH_3)_2$	H	1.195	1.052	1.544	2.07	–
Butobarbital	C_2H_5	$(CH_2)_3CH_3$	H	0.917	0.802	1.170	1.65	–
Pentobarbital	C_2H_5	$CH(CH_3)(CH_2)_2CH_3$	H	1.195	1.035	1.526	2.03***	0.532
Allobarbital	$CHCH=CH_2$	$CHCH=CH_2$	H	0.595	0.544	0.866	1.05***	0.230
Phenobarbital	C_2H_5	C_6H_5	H	0.748	0.693	0.783	1.42	−0.071
Barbital	C_2H_5	C_2H_5	H	0.398	0.368	0.554	0.65	−0.097
Eudan	CH_3	C_6H_5	H	0.602	0.536	0.763	–	–
Hexobarbital	CH_3		CH_3	1.073	0.869	1.310	1.92	0.556
Cyclobarbital	C_2H_5		H	0.938	0.809	1.190	1.86***	0.146
Aprobarbital	C_3H_5	C_3H_7	H	0.748	0.637	–	1.15	0.161
Thiopentobarbital§	C_2H_5	$CH(CH_3)(CH_2)_2CH_3$	H	1.404	–	–	3.00	0.851

* The partition coefficient in the system *n*-octanol–water, taken from Fujita[18].

** The partition coefficient in the system diethyl ether–dimethylformamide–water (2:1:1), taken from Melichai *al.*[20].

*** From Hansch *et al.*[19].

§ In formula 1 the atom of oxygen is substituted by sulphur.

barbiturates as determined by reversed-phase liquid chromatography, R_M (I), R_M (II), and by gas chromatography on 3% NPGS + 0.75% TA, R_M (A), on 3% OV-17, R_M (B), and on 3% OV-1, R_M (C) (Table II). The partition coefficients of barbiturates determined by gas chromatography were calculated also according to eqn. 3.

Eqns. 12–17 give the relationships obtained for fixed phases of varying polarity:

Equation	*n*	*r*	*s*	*No.*
R_M (I) = 0.111 R_M(A) + 0.756	9	0.147	0.287	(12)
R_M (II) = 0.113 R_M(A) + 0.644	9	0.186	0.232	(13)
R_M (I) = 0.289 R_M(B) + 0.537	9	0.370	0.270	(14)
R_M (II) = 0.230 R_M(B) + 0.495	9	0.363	0.220	(15)
R_M (I) = 0.376 R_M(C) + 0.646	9	0.486	0.253	(16)
R_M (II) = 0.310 R_M(C) + 0.575	9	0.494	0.205	(17)

None of the relationships is statistically significant. The value of the regression coefficient increases as the polarity of the fixed phase decreases and it is highest for the non-polar phase OV-1 (eqns. 16 and 17).

In GC, with sufficiently dilute solutions, Henry's law is applicable. The volatility of compounds is then determined mainly by intermolecular forces between the molecules of separated compounds and those of the stationary phase. In contrast, in liquid chromatography there are interactions involving both the stationary and mobile phases. For this reason, GC is considered unsuitable for the determination of partition coefficients that bear a linear relation to the partition coefficients determined in *n*-octanol–water. This conclusion is in agreement with the results of Stoubaut *et al.*[10] who made a similar comparison in O-alkyl O-aryl phenylphosphonothioates. On the other hand, Boček[11] reported that true partition coefficients can be measured in the systems water–nitrogen and oleyl alcohol–nitrogen using the method described by Conder and co-workers[26,27].

TABLE II
PARTITION COEFFICIENTS OF BARBITURATES DETERMINED BY GAS CHROMATOGRAPHY
Conditions as in the text.
Columns: A = 3% NPGS + 0.75% TA on Chromaton N AW DMCS; B = 3% OV-17 on Diatomite CQ; C = 3% OV-1 on Diatomite CQ.

Compound	R_M *value*		
	A	*B*	*C*
Allobarbital	0.895	0.885	0.487
Amobarbital	0.879	1.028	0.565
Aprobarbital	0.788	0.903	0.301
Barbital	0.586	0.669	0.000
Butobarbital	0.827	0.985	0.367
Cyclobarbital	1.507	1.616	1.054
Hexobarbital	0.845	1.404	0.845
Pentobarbital	0.981	1.091	0.636
Phenobarbital	1.710	1.693	1.054

Separation of differently substituted derivatives of urea using reversed-phase liquid chromatography was also studied (see Table III). The R_M values of urea herbicides measured in the mobile phase dioxan–water (1:4) were correlated with Hansch's parameters for anilines[28,29] (Fig. 1). When calculating Hansch's parameter π for anilines with two ring substituents, we took account of the additivity of substitution increments. This simplification was based on the work of Colin *et al.*[17] who for so-called pseudohomologous series of polymethylbenzenes and polymethylphenols obtained quasi-linear relationships between the number of carbon atoms and log k.

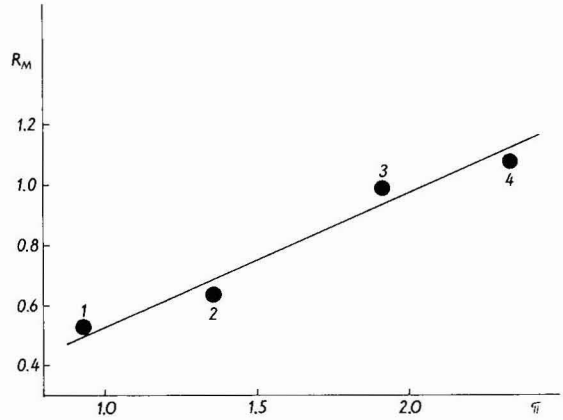

Fig. 1. Relationship between the R_M values of urea herbicides and Hansch's parameter π for anilines: 1 = monolinuron; 2 = metabromuron; 3 = linuron; 4 = chlorbromuron. MicroPak CH-10, dioxan–water (1:4), flow-rate 60 ml/h.

TABLE III
PARTITION COEFFICIENTS OF UREA HERBICIDES DETERMINED BY REVERSED-PHASE LIQUID CHROMATOGRAPHY
Systems: I = MicroPak CH-10, methanol–water (3:7), flow-rate 60 ml/h; II = MicroPak CH-10, dioxan–water (1:4), flow-rate 60 ml/h.

Compound	*Substituents*				R_M *value*		π*
	R_1	R_2	R_3	R_4	*I*	*II*	
Monuron	CH_3	CH_3	H	Cl	0.501	0.367	0.93
Diuron	CH_3	CH_3	Cl	Cl	0.983	0.794	1.91
Monolinuron	CH_3	OCH_3	H	Cl	0.666	0.535	0.93
Chlorbromuron	CH_3	OCH_3	Cl	Br	0.833	1.059	2.34
Methobromuron	CH_3	OCH_3	H	Br	0.760	0.628	1.36
Methoxuron	CH_3	CH_3	Cl	OCH_3	0.412	0.235	1.03
Linuron	CH_3	OCH_3	Cl	Cl	1.135	0.982	1.91

* Hansch's parameter for anilines, taken from Leo *et al.*[28] and Fujita *et al.*[29].

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EFFECT OF ADSORPTION ON THE REPRODUCIBILITY OF RETENTION INDICES OF HYDROCARBONS IN CAPILLARY GAS–LIQUID CHROMATOGRAPHY*

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SUMMARY

The relationship $I = I_0 + a_I \cdot 1/k$ was compared with experimental data for hydrocarbons separated by gas chromatography in capillary columns coated with stationary phases of varying polarity. The influence of adsorption in gas–liquid systems on the reproducibility of retention indices is discussed.

INTRODUCTION

Capillary gas chromatography (GC) has been widely adopted on account of the much higher resolution capacity of capillary columns compared with packed columns. The reproducibility of retention indices measured in various capillary columns in one laboratory and, particularly, in different laboratories has, however, remained unsatisfactory. This fact limits the practical utilization of capillary GC for peak identifications based on retention data, which is important for the characterization of unknown compounds.

There are two ways of overcoming this: either to obtain capillary columns with identical properties or to search for methods that will improve the reproducibility of retention data. Unfortunately, the preparation of capillary columns with identical properties is very complicated and almost impossible in practice.

In this work an attempt has been made to obtain reproducible retention data even under conditions involving adsorption in gas–liquid systems. The separation of hydrocarbons was selected as a model as it is assumed that the problem of reproduc-

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ibility of retention data of hydrocarbons is less serious than with other compounds.

In this paper, as in earlier papers[1–3], it is assumed that the lower reproducibility of the retention data obtained by GC is caused by adsorption interactions of the compounds being chromatographed on gas–stationary phase or stationary phase–column wall interfaces, which are difficult to control. We found a noticeable influence of the thickness of the stationary phase film on retention indices[3]. The problem of adsorption has recently been discussed by Fritz *et al.*[4] and the influence of the thickness of the stationary phase film in glass capillary columns on the retention data has also been investigated[5]. Berezkin[1] proposed the following relationship for retention indices:

$$I = I_0 + a_I \cdot \frac{1}{k} \tag{1}$$

where I_0 is the limiting value of the retention index determined only by the dissolution of the chromatographed compound in the stationary phase, a_I is a constant characterizing adsorption of this compound in the given system (carrier gas–stationary phase–column wall) and k is the capacity ratio of a standard compound, the adsorption of which can be neglected in the given system.

Now, eqn. 1 has been compared with experimental data for the separation of hydrocarbons in glass and metal capillary columns coated with stationary phases of varying polarity. The reproducibility of the I_0 values was evaluated and its dependence on the type of homologous series of the compounds used as reference standards was investigated.

EXPERIMENTAL

Gas chromatographic measurements were performed with a Carlo Erba GI 452 gas chromatograph equipped with a flame-ionization detector.

Stainless-steel capillary columns (50 m × 0.25 mm I.D.) (Laboratory Instruments, Prague, Czechoslovakia) were washed with *n*-hexane and benzene prior to the measurements. They were then coated by the dynamic method with solutions of 1,2,3-tris(cyanoethoxy)propane (TCEP) in acetone of various concentrations in order to obtain capillary columns with various thicknesses of the stationary phase film.

Glass capillary columns were prepared of soft soda-lime glass (Unihost, Glassworks, Jablonec, Czechoslovakia). The glass surface was roughened with methyl trifluoroethyl ether before the columns were coated with Carbowax 20M, and when Apiezon L was used the surface was roughened with concentrated hydrochloric acid and deactivated with trimethylchlorosilane. Stationary phases were coated on the columns by the dynamic method using a mercury piston in such a way that columns with various film thickness were obtained. In order to obtain a thicker film of Carbowax 20M, the surface of one column was covered in advance with barium carbonate. Long glass capillary columns (up to 300 m) were obtained by joining shorter coated columns by means of PTFE tubing[3].

RESULTS AND DISCUSSION

Application of equation 1

Table I gives retention indices for benzene measured at 50°C in stainless-steel capillary columns with various TCEP coatings. Table II gives analogous data for *o*-xylene obtained under the same conditions. These data are shown graphically in Figs. 1 and 2 as the dependences $I = f(k)$ and $I = f(1/k)$.

TABLE I
RETENTION INDICES, CAPACITY RATIOS AND RECIPROCAL CAPACITY RATIOS OF BENZENE ON TCEP COLUMNS AT 50°C

Column	I_{50}^{TCEP}	k	$1/k$
1	770.0	0.03	35.7
2	1014.5	0.25	4.00
3	1049.7	0.69	1.45
4	1060.2	0.42	2.39
5	1109.0	1.01	0.99
6	1111.9	1.04	0.96
7	1124.0	0.97	1.03
8	1146.6	1.87	0.53
9	1169.0	2.50	0.40
10	1180.6	5.12	0.19

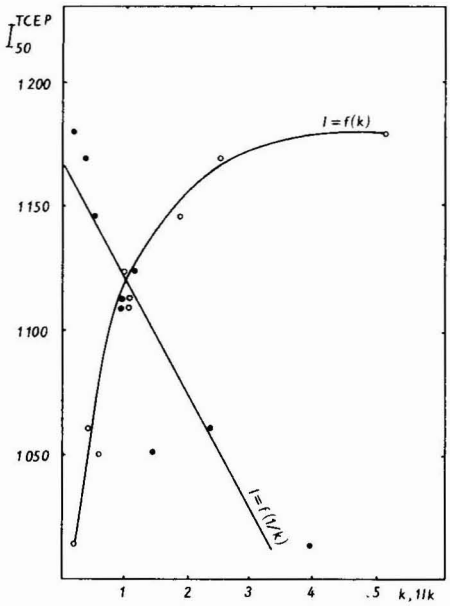
TABLE II
RETENTION INDICES, CAPACITY RATIOS AND RECIPROCAL CAPACITY RATIOS OF *o*-XYLENE ON TCEP COLUMNS AT 50°C

Column	I_{50}^{TCEP}	I_{50}^{TCEP}*	k	$1/k$
1	988.0	–	0.18	5.55
2	1231.5	–	1.22	0.82
3	1249.0	905.5	3.45	0.29
4	1332.2	906.5	5.26	0.19
5	1340.8	905.5	4.30	0.23
6	1364.0	907.0	4.60	0.22
7	1375.4	907.1	9.20	0.11
8	1395.7	908.0	12.10	0.08
9	1398.1	906.6	8.21	0.12
10	1398.8	905.1	8.20	0.12
11	1421.2	908.9	26.70	0.04

* *n*-Alkylbenzene standards.

The experimental results do not disagree substantially with eqn. 1; one experimental point obtained at a small thickness of the stationary phase film (low value of k) is the only exception. Despite this, the limiting values of I_0 were determined with an accuracy that is not sufficiently high. Thus, for benzene $I_0 = 1163 \pm 30$ and $a_I = -42.4 \pm 7.6$ with a regression coefficient $r = 0.90 \pm 0.25$, and for *o*-xylene $I_0 = 1405 \pm 38$ and $a_I = -245 \pm 54$ with $r = 0.84 \pm 0.36$.

Hence the determination of I for *n*-alkanes as standard compounds for the



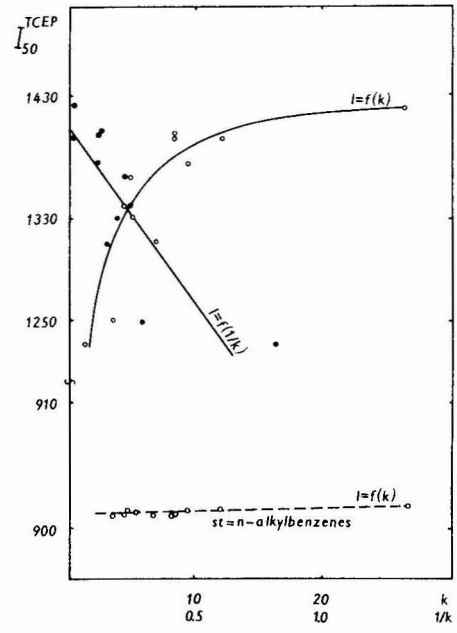


Fig. 1. Dependence of retention index of benzene (*n*-alkanes as standards) on capacity ratio or its reciprocal ($1/k$), obtained by measurement at 50°C in nine metal capillary columns coated with TCEP.

Fig. 2. Dependence of retention index of *o*-xylene (*n*-alkanes or *n*-alkylbenzenes as standards) on capacity ratio or its reciprocal ($1/k$), obtained by measurement at 50°C in ten metal capillary columns coated with TCEP.

determination of Kováts retention indices is not precise enough and reflects the obvious influence of the adsorption of *n*-alkanes on the polar stationary phase. In this connection it is worth noting that a_I is a much larger negative value for *o*-xylene than for benzene. As most of the measurements (eight out of ten) were performed in the same capillary columns, this difference is associated with the use of different *n*-alkane standards for the expression of the retention of *o*-xylene to those used for benzene (adsorption increases with molecular weight).

Influence of homologous standards on the variance of I_0 values

It was shown earlier[1] that the dependence of retention data on the amount of stationary phase in the column can be reduced significantly for polar stationary phases by replacing homologous series of *n*-alkanes with series of other compounds that are readily soluble in the stationary phase. This is why the measurements were performed with the use of homologous series of *n*-alkylbenzenes as standards (Table II). The precision of the determination of I_0 values improved significantly and the value of a_I characterizing the adsorption was reduced substantially in this case: $I_0 = 908.3 \pm 2.0$ and $a_I = -10.4 \pm 4.0$ with $r = 0.68 \pm 0.90$. The results obtained confirm the assumption that adsorption represents a significant contribution to the retention of *n*-alkanes in the column with TCEP.

Table III summarizes the values of I_0 and a_I for isomeric *n*-pentadecenes separated in three glass capillary columns coated with Carbowax 20M and for two types

TABLE III
VALUES OF I_0 AND a_I CALCULATED FOR *n*-PENTADECENE ISOMERS SEPARATED ON CARBOWAX 20M AT 110°C

n-Pentadecene isomer	*n-Alkane standards*				*1-Alkene standards*			
	I_0	s*	a_I	s*	I_0	s*	a_I	s*
cis-7-	1525.4	3.0	−11.7	1.3	1476.8	1.7	1.9	0.74
cis-6-	1528.5	2.9	−12.1	1.3	1480.4	1.6	1.2	0.72
cis-5-	1533.7	4.8	−13.5	2.2	1485.9	0.2	−0.2	0.15
cis-4-	1539.9	4.4	−14.5	2.1	1491.9	0.3	−0.4	0.12
cis-3-	1548.4	5.0	−16.0	2.4	1500.5	0.1	−1.4	0.02
cis-2-	1576.4	7.2	−23.6	4.0	1529.0	2.2	−7.4	1.21
trans-7-	1526.2	3.6	−9.6	1.6	1477.9	1.1	3.8	0.50
trans-6-	1528.2	3.5	−9.7	1.6	1479.6	1.5	3.9	0.69
trans-5-	1531.1	3.5	−10.4	1.6	1482.8	1.4	3.5	0.65
trans-4-	1533.0	3.8	−11.1	1.7	1484.5	0.6	3.0	0.26
trans-3-	1542.6	4.9	−13.6	2.4	1493.7	0.1	1.6	0.04
trans-2-	1562.2	6.4	−18.4	3.4	1514.4	1.7	−2.7	0.89
1-	1547.9	5.0	−14.7	2.5	–	–	–	–

* s = standard deviation.

of standards, *n*-alkanes and 1-alkenes[3]. The application of 1-alkenes made it possible to improve the reproducibility of I_0 values and to reduce a_I values 2–50-fold in the former instance and 3–70-fold in the latter. However, certain characteristic differences in the reproducibility for individual isomers were maintained, but they can be correlated with the structure of the compounds. Thus, not even 1-alkenes as standards for the expression of retention indices eliminated entirely the influence of adsorption on the reproducibility of the retention indices of *n*-alkene isomers on the polar stationary phase. It can be assumed that this effect will increase with increasing polarity of the stationary phase.

Retention indices were measured for *n*-pentadecene isomers depending on the thickness of Carbowax 20M for three groups of homologous standards: *n*-alkanes, 1-alkenes and 1-ethyl-2-alkylbenzenes (Fig. 3). The least variance of the retention indices was obtained for 1-alkenes, *i.e.*, for the compounds most closely related in chemical character to the compounds under analysis. As it is mixtures of compounds of different types that are analysed in practice, the selection of suitable reference substances for polar stationary phases will be problematical (see also ref. 4).

The effect of adsorption on the retention index of an *n*-alkene in capillary columns with a non-polar stationary phase is, as expected, substantially lower. 1-Pentadecene was analysed in four capillary columns coated with Apiezon L and the capacity ratios at 164°C were in the range 0.40–1.87[3]. When *n*-alkanes were used as standards, the value of I_0 was 1489.6 with a standard deviation of 1.0 and the value of a_I was −0.38. The influence of ageing on the retention indices of hydrocarbons, caused by the loss of part of the stationary phase from the column, is for the same reason also substantially less with a non-polar stationary phase[2].

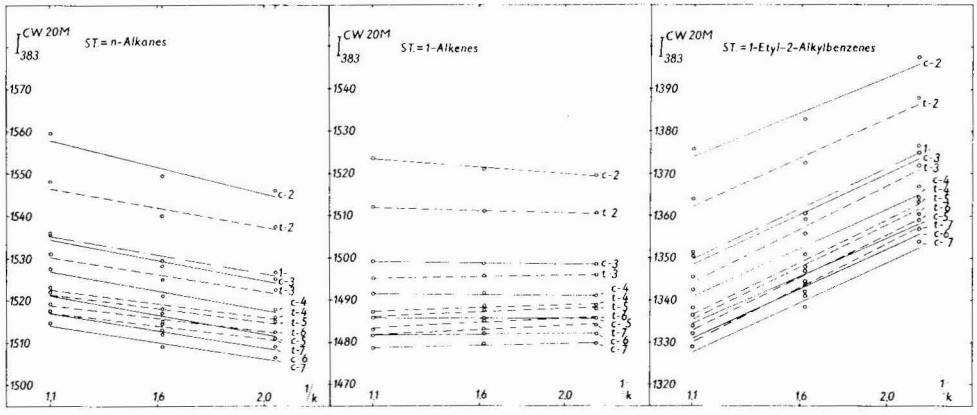


Fig. 3. Dependence of retention indices of all *n*-pentadecene isomers on reciprocal of capacity ratio of 1-ethyl-2-pentylbenzene, obtained by measurement at 110°C in three glass capillary columns coated with Carbowax 20M and with the use of *n*-alkanes, 1-alkenes and 1-ethyl-2-alkylbenzenes as standard compounds for expressing retention indices (*c* = *cis*, *t* = *trans*, 1- = 1-pentadecene).

CONCLUSION

Experimental data for hydrocarbons separated on both polar [1,2,3-tris(cyanoethoxy)propane, Carbowax 20M] and non-polar (Apiezon L) stationary phases appear to be in general agreement with relationship $I = I_0 + a_1 \cdot 1/k$. With the polar stationary phases, the reproducibility of the limiting values of I_0 depends significantly on the type of homologous series of the compounds used as standard substances for the expression of retention indices, with the variance increasing as the adsorption of the standard and the compound under analysis increases. By selecting homologous compounds other than *n*-alkanes, the reproducibility of I_0 values can be improved significantly in certain cases. Retention indices can be obtained with good reproducibility only on stationary phases in which both the analysed and standard compounds dissolve well, *i.e.*, in systems in which adsorption plays a subsidiary role.

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SELECTIVITY OF PARAFFINIC STATIONARY PHASES FOR GAS–LIQUID CHROMATOGRAPHY

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SUMMARY

The selectivities of some paraffinic stationary phases were compared for the non-polar solutes and solutes of low polarity. A linear relationship between the logarithm of relative retention and the reciprocal of the number of methylene groups in the stationary phase molecule was found; this relationship is recommended for use in predicting relative retentions within homologous series of stationary phases. Errors that arise when relative retentions for one paraffinic stationary phase are used to predict those of another are discussed.

INTRODUCTION

The widely used paraffinic stationary phase squalane has zero polarity on the Rohrschneider scale. Other paraffinic stationary phases seem to have the same polarity, but the selectivity of these liquids towards different solutes may differ. Unfortunately, the column temperature that can be used with squalane is restricted, with a maximum of 80–100°C. Many high-molecular-weight liquid paraffins have been proposed for use at higher column temperatures, but they were usually mixtures of polymers with different molecular weights, which precluded the determination of reproducible results. Only one pure paraffinic stationary phase, namely the C_{87} branched-chain paraffin, was found to be a possible standard non-polar stationary phase for gas–liquid chromatography (GLC)[1,2], with an operating range of 40–260°C. The standard non-polar packing was prepared by coating this liquid on a support deactivated by Carbowax 15M[3].

The C_{87} branched-chain paraffin was introduced only recently, so most of the available retention data were determined on different hydrocarbons. It would be useful if these data could be used to predict results with the new standard non-polar stationary phase. No comparisons of the selectivities of paraffinic stationary phases that differ markedly in molecular weight or structure have been reported.

Some papers[4,5] have discussed the influence of molecular weight of paraffinic stationary phases on specific retention volumes (V_g). V_g values, molar heats of solution and activity coefficients for non-polar solutes have been determined in different *n*-paraffins[6–12], but no conclusions about selectivity were drawn. A considerable in-

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fluence of the purity of squalane on selectivity towards solutes of low polarity has been found[13,14], so that published retention data for these solutes on paraffinic stationary phases may be inaccurate.

Therefore, it seems useful to compare paraffinic stationary phases in two steps: (i) comparison of published retention data for non-polar solutes and (ii) consideration of new retention data for solutes of low polarity on purified stationary phases determined under the same experimental conditions. This paper will discuss these two steps.

THEORETICAL

The total selectivity of the stationary phase, r (relative retention), can be considered much more simply when it is divided into two parts, enthalpic and entropic. Relative retention may be expressed as ratio of two partition coefficients (K_L) or specific retention volumes. When the thermodynamic partition coefficient (K) is used, the following equation for molar heat of solution (ΔH_s) and molar entropy of solution (ΔS_s) may be written:

$$-RT \ln K = \Delta H_s - T\Delta S_s \tag{1}$$

where R is the gas constant and T (°K) is the column temperature.

Now,

$$K = \frac{K_L}{A_L RT} \tag{2}$$

where A_L is the amount of stationary phase (moles) per unit volume[15,16]. Taking into account the relationship between K_L and V_g, we can write

$$-RT \ln \left(\frac{V_g A_L}{273.2\ R} \right) = \Delta H_s - T\Delta S_s \tag{3}$$

Eqn. 3 indicates the relationship between V_g and the energy of the intermolecular forces, ΔH_s.

Let us consider the homologous series of n-paraffinic stationary phases. The molecules consist of two different structural groups, methyl and methylene. These groups differ in their energy of dispersion interaction with their environment: the relative values of the dispersion forces[17] of this interaction are 4.65 and 2.05 units when intramolecular shielding is taken into account. Only two methyl groups are present in each n-paraffin molecule (one at each end), and therefore the longer the molecule the weaker are the overall dispersion forces because of the increasing number of methylene groups in the molecule.

The solute molecule is much shorter than the stationary phase molecule and interactions take place between part of the stationary phase molecule and the whole solute molecule. When there is no steric hindrance, the solute molecule has to make contact with the part of the stationary phase molecule that contains a methyl group. However, there is some probability in solution for different groups to make contact.

The longer methylene chain, the lower is the probability that a solute molecule will make contact with a methyl group. Then we can write the following relationship:

$$\Delta H_s \approx 1/n_1 \tag{4}$$

where n_1 is the number of methylene groups in the stationary phase molecule.

The validity of eqn. 4 may be proved by plotting experimental ln V_g values *versus* $1/n_1$ (if it is assumed that ln V_g depends mainly on ΔH_s for the case concerned). Fig. 1 shows that eqn. 4 is valid for each series of the experiments; because of poor reproducibility of V_g, the values obtained in different laboratories are not identical. When discussing the data in Fig. 1 one must take into account that the stationary phases relating to lines 1 and 2 relate to branched-chain hydrocarbons.

Relative retention data have much better inter-laboratory reproducibility than V_g, and therefore it would be preferable to consider relative retention as a measure of the selectivity of stationary phases:

$$-RT \ln r = \Delta H_s^\circ - T\Delta S_s^\circ \tag{5}$$

where ΔH_s° and ΔS_s° are relative values of the functions. However, the relative retentions are more difficult to consider because the retentions of two solutes have to be taken into account.

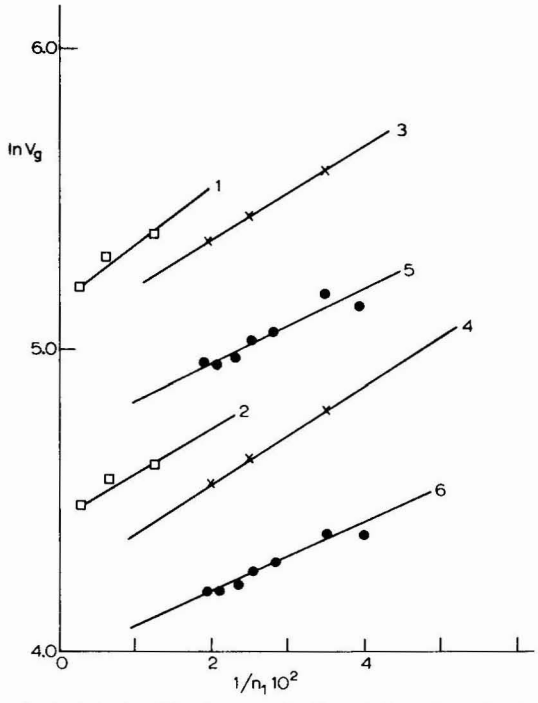

Fig. 1. Relationships between ln V_g and $1/n_1$. Data for lines 1–4 were calculated from ref. 4 and those for lines 5 and 6 from ref. 6. Lines 1 and 3 = *n*-nonane; 2, 4 and 5 = *n*-octane; and 6 = *n*-heptane. Column temperature, 100°C.

Generally, isoparaffins are to be preferred to *n*-paraffins as stationary phases for GLC because they have lower melting points and therefore a wider operating range. For example, the C_{87} branched-chain paraffinic stationary phase has a melting point of about 35°C with a maximum column temperature of 260°C. However, some retention data have been determined employing *n*-paraffin stationary phases, especially for use in theoretical studies.

It is useful also to compare the selectivities of different isomeric stationary phases. Theoretical predictions about the influence of structural changes of the stationary phase molecule on the selectivity are impossible because no information exists about the real conformation of the molecules in solution. Therefore, it is desirable to consider both reported retention data and new data together in order to establish variations for different paraffinic stationary phases.

EXPERIMENTAL

Reagents and apparatus

Retention data were determined by using a Varian Model 1860 gas chromatograph equipped with a flame-ionization detector (FID). The stainless-steel column (1.5 m × 3 mm I.D.) was packed with 10% of the stationary phase on Chromaton N Super (Lachema, Brno, Czechoslovakia), 0.20–0.25 mm. All freshly prepared packings were conditioned for 15 h at the maximum column temperature. Helium was used as the carrier gas.

The solutes were injected by gas or liquid syringes depending on the boiling point. When liquids were injected the injector temperature was maintained 20°C higher than the column temperature. Retention data were determined at column temperatures from 35 to 60°C.

C_{19}, C_{21}, C_{23} and C_{24} *n*-paraffins and squalane were purified by passage through a silica gel column. *n*-Hexane (the standard), *n*-heptane, cyclohexane, 1-heptene, benzene, chloroform and dichloroethane were used as the solutes. Only single solutes were injected.

Calculations

Methane was used for dead time measurements. The ln r values were plotted against $1/T$ for four or five column temperatures and the smoothed r value was calculated for a standard temperature of 50°C. The relative molar heat of solution was calculated graphically by using the equation

$$\Delta H_s^\circ = \frac{R\,(\ln r_1 - \ln r_2)}{1/T_1 - 1/T_2} \tag{6}$$

where the subscripts 1 and 2 refer to different column temperatures.

The mean relative standard deviation of the r value was about 0.1% and that of ΔH_s° was 3%.

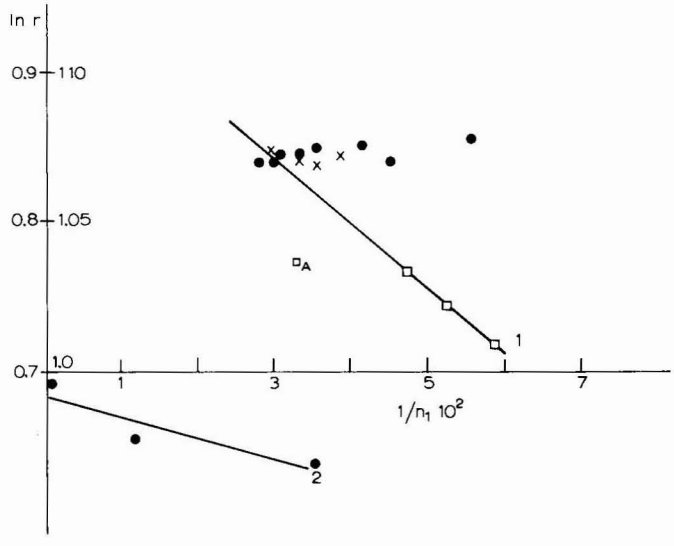

Fig. 2. Relationship between ln r and $1/n_1$ for (with n-hexane as the standard and n-heptane as the solute). Point A refers to squalane. ●, Calculated from ref. 12; ×, calculated from ref. 6 (ln r scale 0.7–0.9). Two lines (1 and 2) were constructed from original data (ln r scale 0.95–1.10). For line 2 three branched-chain paraffin stationary phases were used (see text)[18].

RESULTS AND DISCUSSION

Paraffinic solutes

The relationship between the r value of n-heptane (with n-hexane as standard) and $1/n_1$ is shown in Fig. 2. The n_1 value for squalane (as for other branched-chain paraffins) was calculated by subtracting the contributions due to two carbon atoms in the molecule. The experimental points taken from refs. 6 and 12 show no clear correlation. The original points for C_{19}–C_{23} n-paraffins lie on a common line without marked deviations. Relative retention increases proportionally to $1/n_1$; hence the slopes of ln V_g and ln r *versus* $1/n_1$ are of opposite sign. The increase in the density of the homologous series of n-paraffins seems to be the main reason for the increase in the selectivity of the stationary phases.

The point for squalane (A) is below the line for the n-paraffins, which indicates clearly the influence of molecular structure on selectivity. The three high-molecular-weight stationary phases used (squalane, C_{87} and polyisobutylene, molecular weight 2000; data from ref. 18) have different molecular structures, which explains the deviation of the points from the common line (line 2).

The data in Fig. 2 allow one to predict changes in relative retentions for n-paraffins from one stationary phase to another. For example, the relative retentions on the C_{30} n-paraffin and squalane differ by 2% and those on the C_{30} and C_{40} n-paraffins by 1.2%.

A similar relationship is observed for relative molar heats of solution (Fig. 3). A slight decrease in ΔH_s° is seen for the original data. About the same variations are obtained for the entropic selectivity[19]: the values are -0.95, -1.006, -1.011 and -0.937 for the C_{19}, C_{21} and C_{23} n-paraffins and squalane, respectively. On comparing

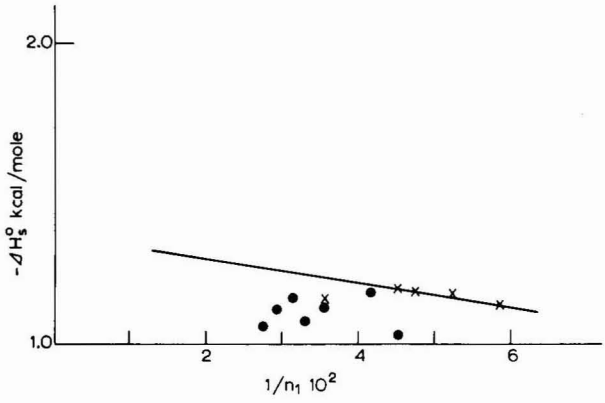

Fig. 3. Relationship between ΔH_s° for *n*-heptane and $1/n_1$ (with *n*-hexane as the standard). ×, New data; ●, data from ref. 12.

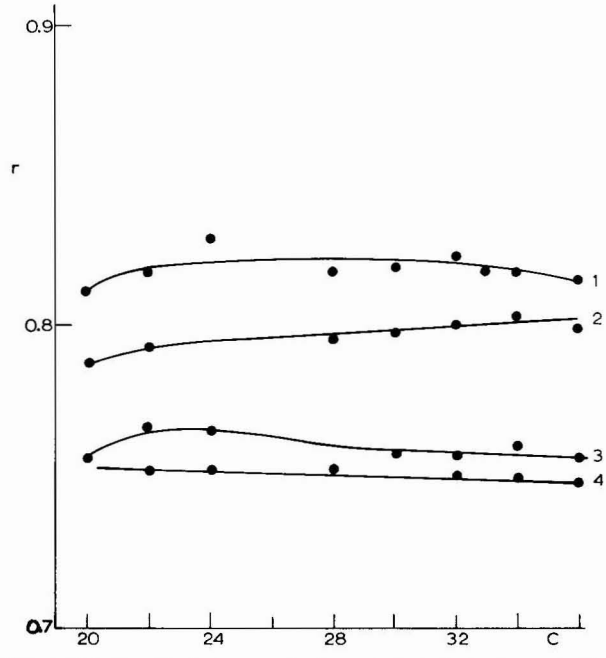

Fig. 4. Relationship between *r* for branched-chain heptanes (with *n*-heptane as the standard) and number of carbon atoms in the *n*-paraffin stationary phase molecule. 1, 3-Methylhexane; 2, 2,3-dimethylpentane; 3, 2,4-dimethylpentane; 4, 2-methylhexane.

the influences of ΔH_s° and the entropic selectivity it can be concluded that ΔH_s° is the dominating factor.

The relative retentions for branched-chain heptanes (with *n*-heptane as the standard) (Fig. 4) were calculated from refs. 6 and 12. No clear relationships were observed for the solutes, the *r* values deviating by 1.5–3 %. On comparing these data with the relative retentions for *n*-paraffins it seems that the trends and deviations are similar.

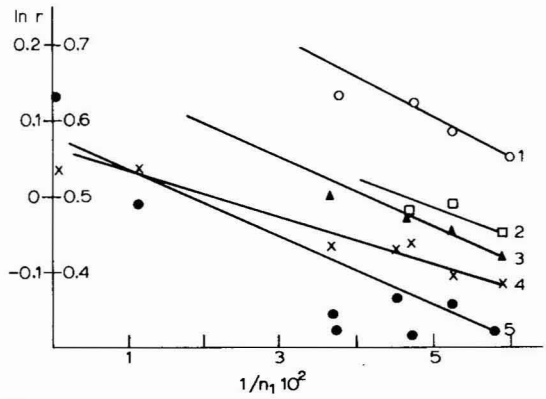

Fig. 5. Relationship between ln r and $1/n_1$ (with n-hexane as the standard) for (1) cyclohexane, (2) heptene-1, (3) dichloroethane, (4) benzene and (5) chloroform. The ln r scale -0.1 to 0.2 relates to lines 3 and 5; scale 0.4–0.7 relates to lines 1 and 4; line 2 lies between ln r values of 0.8 and 0.9.

Solutes of low polarity

Fig. 5 indicates a general tendency for r to increase with decreasing $1/n_1$, corresponding to the relationship for n-paraffins. Thus, this relationship appears to be generally applicable to solutes with different molecular weights and polarities. It should be noted that for solutes of low polarity some deviations from the common line are observed so that the precise slope cannot be evaluated. To a first approximation, the slope depends mainly on the geometrical form of the solute molecules (see the data for benzene and cyclohexane).

Some relative molar heats of solution for the solutes of low polarity are plotted *versus* $1/n_1$ in Fig. 6. A general correlation of ln r and ΔH_s° with $1/n_1$ is observed in Figs. 5 and 6. On increasing the molecular weight of the stationary phase, the relative retentions increase also.

Interesting data were reported recently[20] on a comparison of squalane and C_{87} stationary phases for the separation of pentadecene isomers. It was shown that for many isomers the Kováts retention indices are 0.1–0.7 units lower on C_{87} than on squalane; for only three isomers were the retention indices greater (by 0.1–0.4

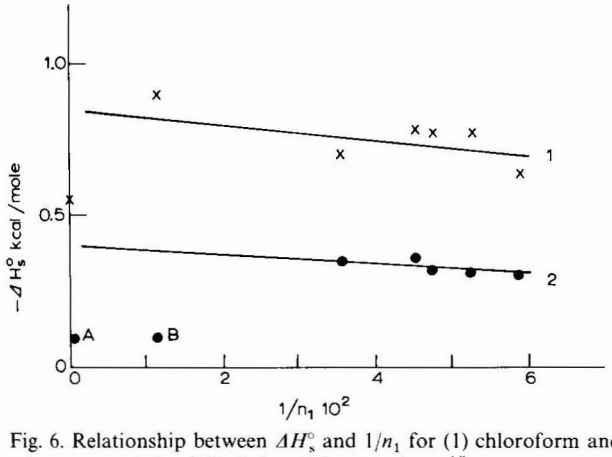

Fig. 6. Relationship between ΔH_s° and $1/n_1$ for (1) chloroform and (2) benzene. Points A and B refer to columns containing 5% of the stationary phases[18].

units) on C_{87}. These data for olefins illustrate the limits of the errors when the retention data for squalane are used to calculate those for C_{87} (about 1.5 units for the case concerned).

CONCLUSIONS

The relationships presented here allow some conclusions to be drawn about the calculation of the retention data for one paraffinic stationary phase from those for another. The most important practical problem is the calculation of hydrocarbon retention data for C_{87} from values for squalane. Data for *n*-paraffins indicate that the relative retentions of paraffinic solutes differ by 1.6% between these two stationary phases, which corresponds to 0.7 Kováts retention index unit. This value is less than the inter-laboratory reproducibility of the determination of paraffin retention data on a non-polar stationary phase. Thus, the retention data for isoparaffins on squalane may be transferred to C_{87} by taking into account the slightly greater retention indices (about 0.5 retention index unit) for C_{87}. This is of great importance because retention data for isoparaffins on squalane are available for isomers up to C_{10}.

Other classes of hydrocarbons show greater changes in relative retention between squalane and C_{87}. For example, the relative retention of benzene is 9.5% higher on the latter. Similar results were obtained for solutes of low polarity. Therefore, preliminary experiments are necessary for each class of solutes in order to establish whether it is possible to predict retention data for C_{87} from those for squalane.

When using relative retentions within homologous series of the paraffinic stationary phases to predict those of other members, it is reasonable to use the relationship between $\ln r$ and $1/n_1$ (Figs. 2 and 5). Data for squalane are always below the common line for *n*-paraffinic stationary phases.

The results presented emphasize once again that even paraffinic stationary phases must be very pure products and technical mixtures similar to hydrogenated Apiezons cannot be recommended for use in GLC.

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APPLICATION OF A COPPER TUBULAR ELECTRODE AS A POTENTIOMETRIC DETECTOR IN THE DETERMINATION OF AMINO ACIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A copper tubular electrode (CTE) has been used as a potentiometric detector in the determination of amino acids by reversed-phase high-performance liquid chromatography. The reference electrode was an electrically grounded tubular platinum electrode inserted in the flow stream. Comparison with a variable-wavelength UV absorbance detector showed that the CTE gave comparable response time and sensitivity, but was much more selective, showing response only to copper-binding molecules. The CTE has been applied to the analysis of urine and an intravenous amino acid preparation; no sample pre-treatment was required, other than filtration. Microgram quantities of amino acids were readily detected by using the CTE.

INTRODUCTION

Various detection systems have been used for the determination of amino acids by high-performance liquid chromatography (HPLC). Detection by UV absorbance at the usual wavelength of 254 nm is not optimal for amino acids (due to their low absorptivity), and it is necessary to employ a wavelength of 200 nm for satisfactory results[1]. Use of such a short wavelength introduces problems of solvent purity. More sensitive detection may be achieved by using the fluorescence or chemiluminescence properties of amino acid derivatives, particularly 5-dimethylaminonaphthalene-1-sulphonyl (Dns) derivatives[2,3] formed by use of pre- or post-column reactions.

The capacity of amino acids to form complexes with metal ions is well recognised[4] and has been applied to HPLC through the use of a copper-selective membrane electrode for detection in the analysis of amino acids[5]. Amino acids were indirectly detected by monitoring the reduction in free copper ion activity resulting from post-column reaction between the eluted amino acids and an added copper ion solution. This system, although sensitive, has a number of inherent disadvantages, including peak broadening resulting from the excessive post-column volume introduced by the reaction coil, slow electrode response and a high susceptibility of the electrode to poisoning by complexing agents[6,7].

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In this paper, we describe the use of a copper tubular electrode (CTE) as a sensitive and selective detector in the analysis of amino acids by HPLC. Alexander and Maitra[8] have recently reported the use of copper wire and CTE electrodes as sensitive universal detectors in continuous-flow systems, in which they were found to be superior to the copper-selective membrane electrode for direct quantitation of amino acids. This system is shown to be useful as a detector for amino acids without post-column reaction of the eluted amino acids with copper ions.

EXPERIMENTAL

Instrumentation

A schematic diagram of the flow system used is shown in Fig. 1. In this system, a Waters Assoc. (Milford, MA, U.S.A.) Model 6000 solvent pump, a Waters Model U6K injector, a Waters Model 450 variable-wavelength detector and the electrode detector were linked serially in flow to each other. The electrode detector (see Fig. 2) consisted of two tubular electrodes, one of copper and one of platinum, both of which were connected to an Activon voltage offset controller (± 1.5 v), and the platinum electrode was grounded. The outputs from the UV detector and the voltage controller were linked to a two-pen Omniscribe Model B5271 recorder, which had a 10-mV input.

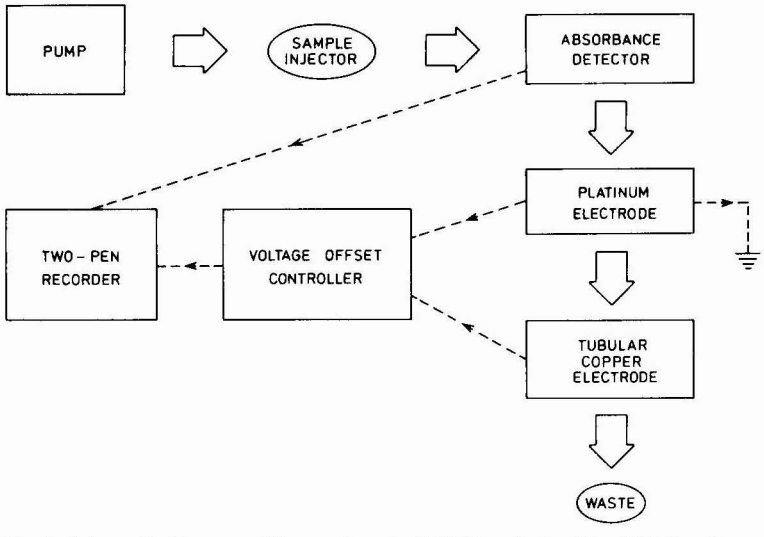

Fig. 1. Schematic diagram of flow system for HPLC analysis with a UV absorbance detector in series with the platinum reference electrode and the CTE: The arrows represent the flow-path; — — — represent electrical connections.

The components of the electrode detector system were as follows. The internal-flow CTE was constructed from copper rod 0.5 cm in diameter and 2 cm in length. This rod was precisely drilled to give an I.D. of 0.75 mm for the flow-path, the length of which was varied over the range 0.5–5.0 mm (1 mm was the most frequently used). The platinum electrode was a 1-cm length of platinum tubing (0.75 mm I.D.) sand-

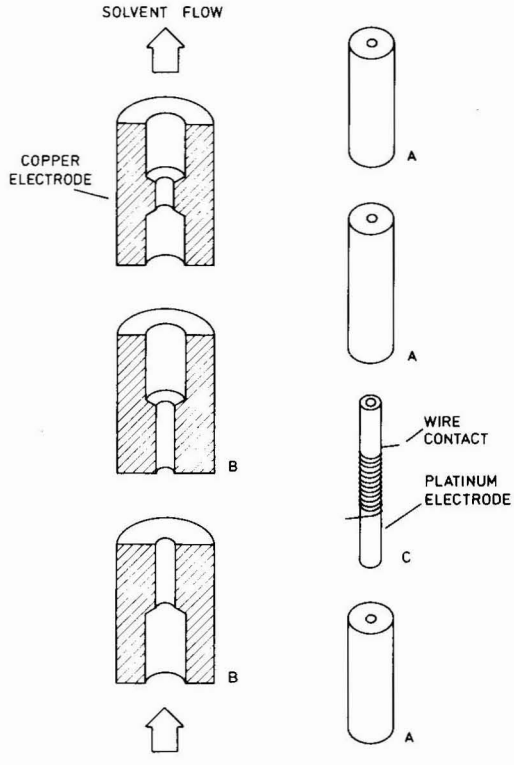

Fig. 2. Diagram showing construction of the CTE and platinum reference electrode. A = PTFE Flow tubing; B = PTFE moulding; C = platinum electrode.

wiched between two pieces of PTFE tubing (see Fig. 3), which served both mechanically to support the platinum electrode and to provide a means of attaching the flow tubing used to connect the various components of the system.

Reagents and stock solutions

Amino acids were obtained from various sources: glycine from BDH (Poole, Great Britain); valine from Koch-Light (Colnbrook, Great Britain); *l*-isoleucine from

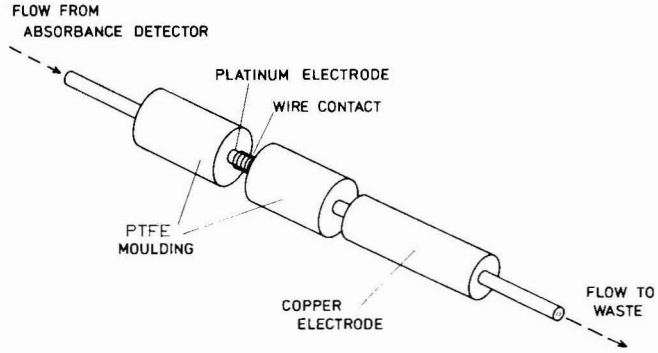

Fig. 3. System used for connecting the platinum reference electrode and the CTE.

BDH; methionine from NBC (Cleveland, OH, U.S.A.) and phenylalanine from Merck (Darmstadt, G.F.R.). The amino acids were used without further purification, and standard solutions were prepared immediately before use by dissolving weighed amounts in a buffer solution (pH 6.7) prepared from AR-grade sodium hydroxide and potassium dihydrogen phosphate (BDH).

Volucon standard buffers were used to calibrate the pH meter.

The mobile phase used for the HPLC separation of amino acids was prepared by mixing 50 ml of 1 *M* NaH_2PO_4, 19.2 ml of 1 *M* NaOH and 10.0 ml of 40% (w/v) formaldehyde in a 1-l volumetric flask; the solution was then diluted to the mark to give a final pH of 6.7 $\pm$ 0.1. All water used for the chromatographic procedure was distilled and passed through a Millipore Q water-purification system before use, and methanol was triply distilled in all-glass apparatus.

The urine control sample was obtained from Travenol Labs. (Costa Mesa, CA, U.S.A.) and was freshly reconstituted before use. The pharmaceutical intravenous amino acid solution was also obtained from Travenol, under the trade name "Synthamin 17", and contained nine essential and six non-essential amino acids in concentrations ranging from 400 mg to 21 g per litre; this solution was diluted by a factor of 25 for chromatographic runs. Both urine and the intravenous solution were filtered through a 2.5-μm Millipore filter before injection into the chromatograph.

Chromatographic procedure

Separation by HPLC was accomplished at 25°C on a μBondapak C_{18} column (30 cm $\times$ 3.9 mm I.D.; Waters Assoc.). The column was standardised by using the manufacturer's recommended procedure[9] and gave counts in the region of 4000 theoretical plates. The mobile-phase flow-rate was set at 1 ml/min (unless otherwise stated), producing a back pressure of 1000 p.s.i. The absorbance detector was operated at 200 nm with a sensitivity setting of 0.1 a.u.f.s.

In this study, all eluted compounds were detected both by the UV detector and the CTE, and the peaks were displayed on the two-pen recorder (chart speed of 1 cm/min). Each data point shown represents the mean of triplicate injections.

To prevent a decrease in sensitivity of the CTE due to poisoning, the electrode surface was periodically regenerated by rapid flushing with 5 ml of 8 *M* HNO_3 followed by 20 ml of distilled water and 20 ml of methanol. When the electrode was stored in the flow system, a solvent consisting of methanol–water (50:50, v/v) adjusted to pH $\approx$5 was used to prevent deterioration of the electrode.

RESULTS AND DISCUSSION

Mobile phase

The effect of pH on the response of the CTE was discussed in a previous paper[8], wherein it was shown that sensitivity for amino acid detection was greatest at high pH values. In this work, the optimum pH for the mobile phase was 6.7, which represented a compromise between maximum sensitivity and the prevention of electrode poisoning.

Fig. 4 shows a typical chromatogram of a separation of five amino acids with the CTE as detector.

A slightly acidic mobile phase was necessary to discourage the formation of

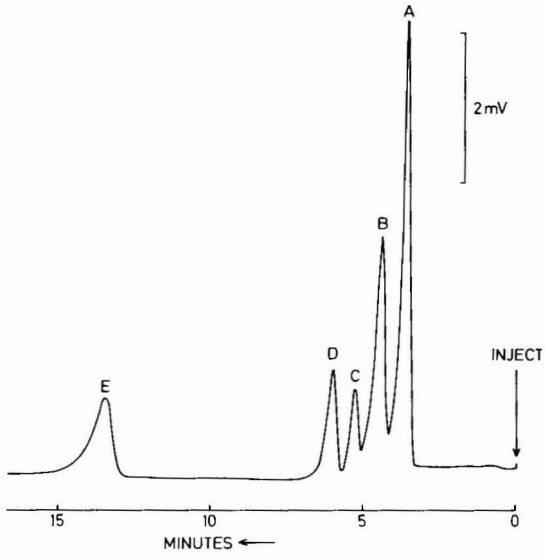

Fig. 4. Chromatogram of an amino acid mixture (1 μl) injected into a reversed-phase column coupled to the electrode detector system. Peaks: A = glycine (6.6 μg); B = valine (5.2 μg); C = methionine (2.8 μg); D = isoleucine (3.9 μg); E = phenylalanine (1.9 μg).

insoluble cupric hydroxides, carbonates and phosphates on the inner surface of the CTE. The ready formation of such compounds in alkaline media would result in potential drift, leading to error in the measured concentration[10].

The purpose of formaldehyde in the mobile phase was to repress negative deviation of the baseline, which occurred most prominently before the peaks due to such sulphur-containing amino acids as methionine and cysteine. The exact mechanism whereby formaldehyde can eliminate such negative baseline deviations is not clear, but its reducing properties may assist in improving the rate of exchange of the amino acids on the electrode surface. It is known[8,10] that the mild reducing properties of formaldehyde prevent oxide film formation at electrode surfaces, and it was added to the mobile phase for the same reasons as previously reported[8,10] for use with copper membrane electrodes, that is, to give baseline stability. Formaldehyde did not affect the amino acid peak heights as shown in Fig. 4.

Reference electrode

The platinum tubular electrode shown in Figs. 1, 2 and 3 was used for two purposes. First, it acted as an auxiliary electrode to reduce electrical noise; secondly, it served as a reference electrode to the CTE. Since it was electrically grounded, the reference used was the earth potential. This configuration was adopted only after considerable experimentation with conventional reference electrodes; however, we found that the electrically grounded platinum electrode gave greatest sensitivity and least baseline noise.

Electrode flow-path

With glycine as representative amino acid, the electrode response was studied

as a function of the length of the electrode flow-path for solutions of three different concentrations of glycine (5.8, 11.5 and 17.4 $\mu g/\mu l$). In all instances, the electrode signal reached a maximum for a flow-path of 1.0 mm.

Poor response for very short flow-paths is considered to be due to the relatively short residence times of the amino acid ligands in the electrode, whereas reduced response in the longer-path-length electrodes is due to sample-dispersion effects. The optimum flow-path length of 1 mm was adopted in all further work.

Flow-rate

The effect of flow-rate on response time and sensitivity was studied by removing the HPLC column, replacing it with a suitable connector and then injecting glycine solution (3.4 $\mu g/\mu l$) at various flow-rates. Values of $t_{max.}$, the time required to reach the maximum of the chromatographic peak, were measured for each flow-rate; the results are shown in Table I.

TABLE I
RESPONSE CHARACTERISTICS OF THE CTE AND ABSORBANCE DETECTORS AT VARIOUS FLOW-RATES

Flow-rate (ml/min)	*Peak height (mm)*		$t_{max.}$ *(sec)*	
	UV detector	*CTE*	*UV detector*	*CTE*
0.5	122	98	24.2	27.0
1.0	90	88	13.3	15.8
2.0	57	74	6.9	8.5
3.0	42	63	4.6	5.5
4.0	37	58	3.5	4.0

In agreement with our earlier findings[8], the CTE response improved with increasing flow-rate; however, this was accompanied by a decrease in sensitivity due to the decreased residence time of the amino acid in the electrode. The response time of the CTE compared favourably with that of the absorbance detector, whereas the sensitivity of the CTE (as measured by peak height) was less dependent on flow-rate than was that of the absorbance detector. It is clear that the optimum flow-rate would represent a compromise between response and sensitivity; in this work we used a flow-rate of 1.0 ml/min.

The relationship between flow-rate and resolution was also examined to provide additional information on the response of the CTE. Injections of 1 μl of a solution containing 11.5 $\mu g/\mu l$ of glycine and 8.8 $\mu g/\mu l$ of isoleucine were made at various values of solvent flow-rate and the resolution (R_s) of the two resulting peaks was calculated by using the conventional formula[11]. For values of $R_s < 0.8$, peaks are considered to be only partially resolved, whereas $R_s > 2$ indicates resolution with at least two peak-base widths between peak maxima. The results are shown in Fig. 5.

A decrease in resolution with increasing flow-rate was observed for both the absorbance detector and the CTE, but the resolution achieved with the absorbance detector declined much more rapidly than for the CTE. It is noteworthy, however, that, at all flow-rates tested, the UV detector gave slightly superior resolution.

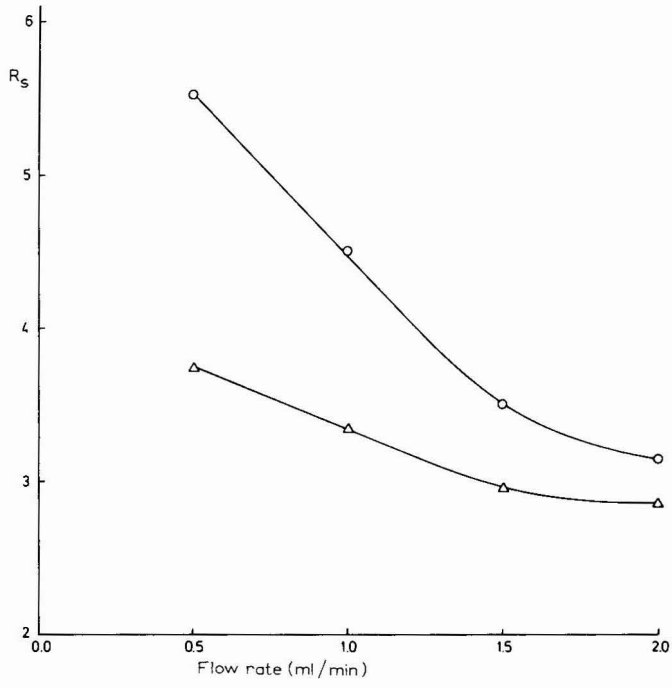

Fig. 5. Resolution (R_s) of glycine and isoleucine at different flow-rates obtained using the electrode detector (△) and the UV absorbance detector (○).

Calibration

Calibration curves for glycine, valine and isoleucine were prepared by using both the absorbance detector and the CTE. Linear plots were obtained with the UV detector for the three amino acids in the concentration range 0–25 μg/μl. The calibration plots obtained for the CTE were non-linear and are shown in Fig. 6; the electrode response follows the elution order glycine, valine, isoleucine. The shapes of the CTE calibration plots are similar to those obtained by Loscombe *et al.*[5] in their work with a copper-selective membrane electrode.

The precision of the electrode response was estimated by 10 replicate injections of a solution containing glycine and isoleucine at concentrations of 1.74 and 1.30 μg/μl, respectively. Coefficients of variation of 1.4% for glycine and 2.5% for isoleucine were obtained; these results compared favourably with those obtained by using the absorbance detector, which gave coefficients of variation of 4.0 and 3.6%, respectively, for the same 10 injections.

By using the definition of detection limit as three times the baseline noise, the calculated detection limits for glycine, valine and isoleucine were 75, 200 and 300 ng, respectively, in a 1-μl injection.

We are currently developing a combined recorder offset and signal-amplification system that will permit detection of smaller amounts of amino acids than stated above. Initial studies have shown that a ten-fold increase in sensitivity over the above values can be easily attained.

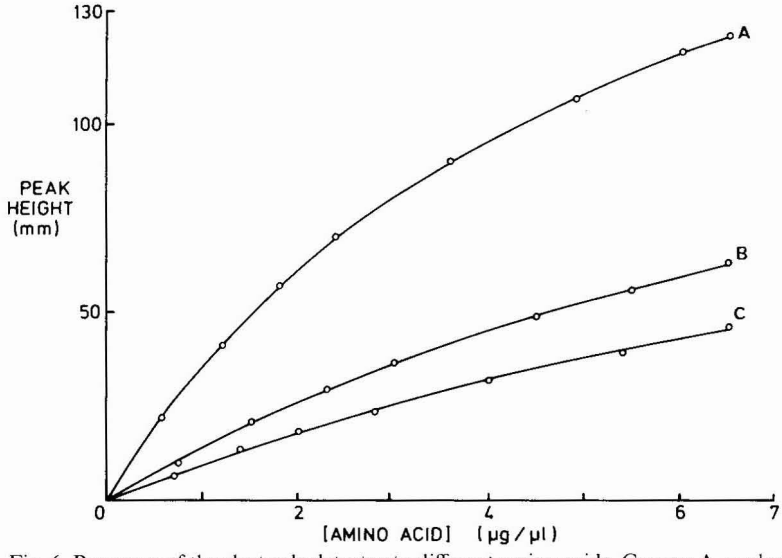

Fig. 6. Response of the electrode detector to different amino acids. Curves: A = glycine; B = valine; C = isoleucine.

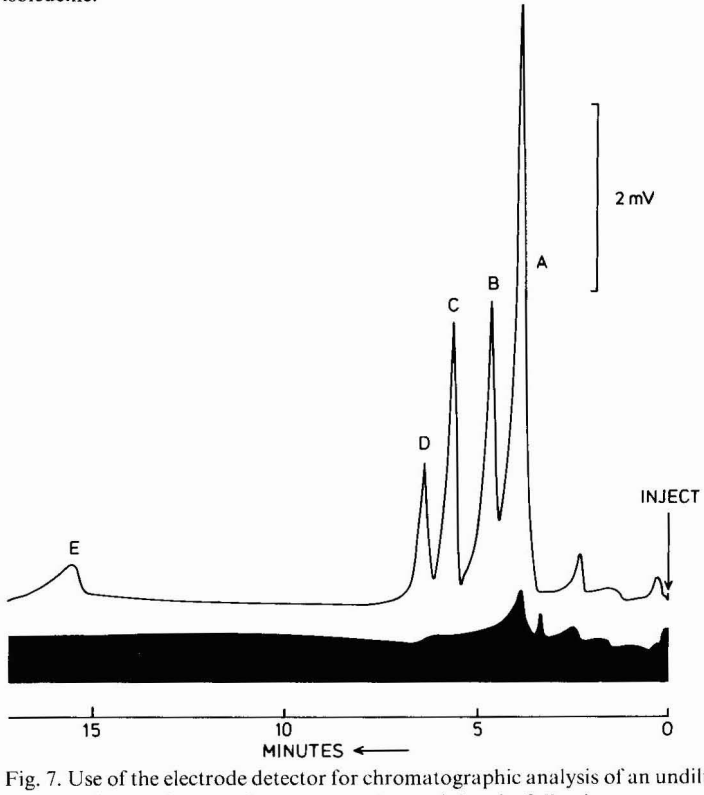

Fig. 7. Use of the electrode detector for chromatographic analysis of an undiluted urine sample (dark trace) and a "spiked" urine sample (upper trace) containing the following amounts of amino acids added per μl of urine: A = glycine (7.5 μg); B = valine (5.3 μg); C = methionine (7.6 μg); D = isoleucine (4.1 μg); E = phenylalanine (0.7 μg). Conditions: 1-μl injection; flow-rate 1 ml/min.

APPLICATIONS

The suitability of the CTE for analysis of urine and a pharmaceutical preparation was investigated. The freshly reconstituted urine sample was not pre-treated in any way except for filtration (Millipore filter) before injection. The chromatograms obtained for the urine sample using both detectors are shown in Figs. 7 and 8; these figures also show the chromatograms obtained from urine with five amino acids added.

The chromatograms obtained by using the UV detector are characterised by a profusion of unidentified peaks, and this background renders recovery calculations of

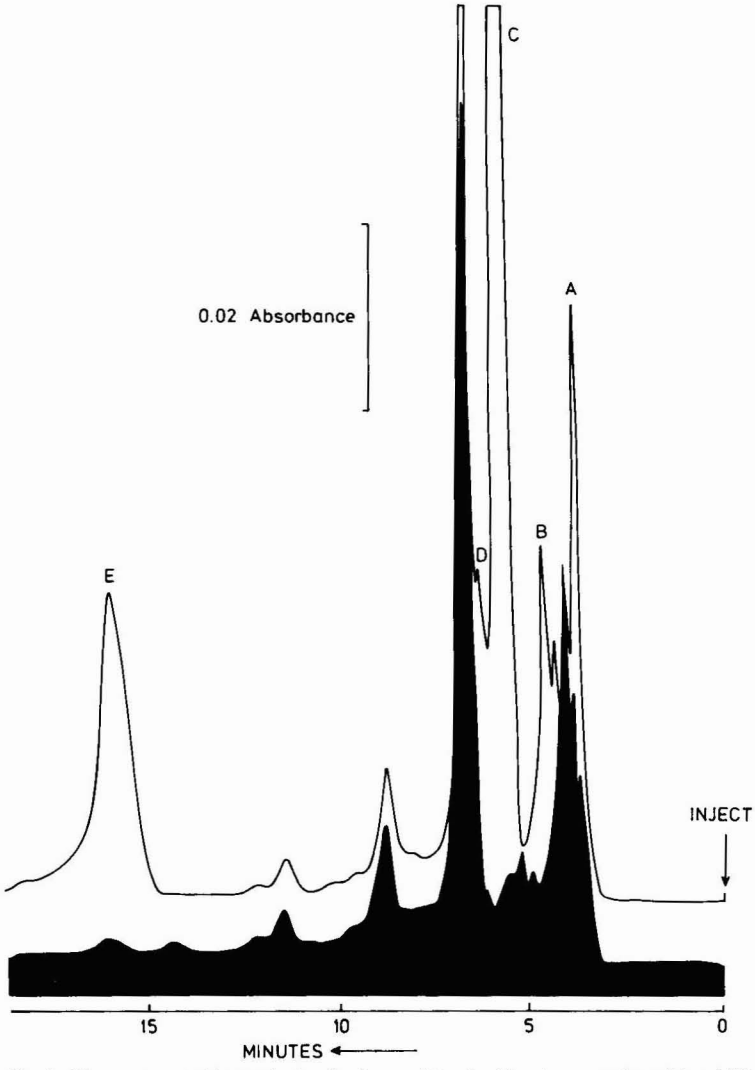

Fig. 8. Chromatographic analysis of urine and "spiked" urine samples with a UV absorbance detector (at 200 nm) under conditions shown in Fig. 7.

the added amino acids difficult. In contrast, the CTE chromatogram of the blank urine sample is very "clean", and the peaks produced by the added amino acids are easily identified. From these chromatograms, the recoveries for glycine, valine, isoleucine, methionine and phenylalanine were 96, 106, 107, 105 and 100%, respectively. The amounts of amino acids added to the sample are shown in the figures. Fig. 7 illustrates the main advantage of the CTE, that is, its selectivity, which eliminates the need for major pre-treatment of the sample.

An intravenous solution containing 15 amino acids and excipients (such as sodium metabisulphite, sodium acetate and sodium chloride) was analysed using the CTE detector; no pre-treatment of the sample was involved, except dilution and filtration. The chromatogram obtained is shown in Fig. 9. No attempt was made to identify or quantify each amino acid, and the separation conditions were not optimised, as the purpose was merely to demonstrate the utility of the CTE for pharmaceutical analysis. However, peaks due to 11 of the 15 components can be discerned, and it is likely that the remaining amino acids are eluted together with the resolved components.

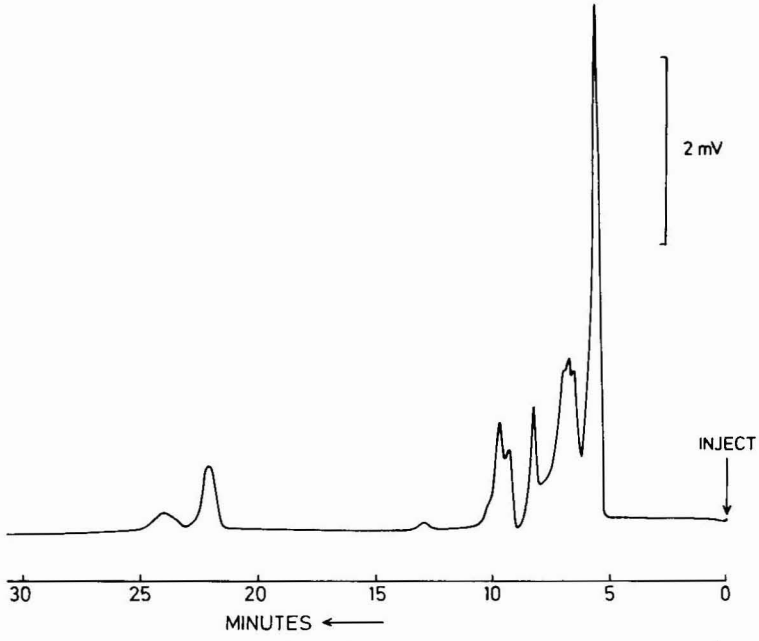

Fig. 9. Chromatographic analysis of an amino acid intravenous solution with the electrode detector. Conditions: 1-μl injection; flow-rate 1 ml/min.

CONCLUSIONS

This study has demonstrated that the CTE can be used as a selective detector in HPLC. In using such an electrode, problems associated with post-column derivatisation procedures or absorbance detection of solutes having poor UV absorption are minimised. The performance of the CTE, as indicated by its response time and sensitivity, is comparable with that of the UV absorbance detector. The major advantages

of the CTE are: first, the selectivity of the CTE is greatly superior to UV detection, and secondly the CTE can be manufactured at very low cost.

In this paper, amino acids have been used as solutes to demonstrate the application of the CTE to HPLC. Other copper-binding compounds may also be detectable. Narang and Gupta[12] have shown that sulpha drugs (sulphanilamide, sulphaguanidine, sulphathiazole, sulphamerazine, sulphadiazine and sulphapyridine) readily form complexes with copper, and Evans *et al.*[13], in their discussion of the analysis of porphyrins in biological materials by HPLC, have reported that copro- and meso-porphyrin esters readily complexed copper. In this laboratory, it has been found that the CTE responds to a variety of solutes. Further, this study opens up interesting possibilities of the use of tubular electrodes constructed from other metals as selective potentiometric detectors for HPLC systems. Further work on this aspect is in progress.

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USE OF MIXED LITHIUM–SODIUM BUFFERS AND POTASSIUM BUFFERS FOR SEPARATION OF THE COMMON FREE AMINO ACIDS IN PLANT MATERIAL ON A TWO-COLUMN SYSTEM

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SUMMARY

A method for the determination of amino acids in plant extracts using a two-column system is described. The acidic and neutral amino acids are separated on a column of Beckman AA-15 resin, with three buffers of mixed lithium citrate–sodium citrate. With the mixed buffers the back pressure on the column was lower than with a pure lithium buffer, but glutamine and asparagine were still well separated from the other amino acids. The basic amino acids and some related compounds are separated on a column of Beckman PA-35 resin, with two buffers of potassium citrate.

INTRODUCTION

The use of lithium citrate buffers has made it possible to separate the amides asparagine and glutamine from each other and from the other amino acids in ion-exchange chromatography[1]. Many modifications of this method have been described[2–7]. These amides have also been separated by use of sodium buffers and temperature programming[8], but at the expense of the separation of aspartic acid and serine. The use of lithium buffers results in a higher column pressure and hence a higher risk of leakage at the connections and valves in the analyzer than experienced with sodium buffers.

Owing to the increased destruction of glutamine at elevated column temperatures[9], it is necessary to keep the column temperature as low as possible, but this results in a higher column pressure. In most of the analytical systems previously described a column temperature of 35–40°C is used until glutamine is eluted from the column.

This paper describes a method for the separation of acidic and neutral amino acids on a long column using mixed lithium citrate–sodium citrate buffers to overcome the pressure problem while maintaining a satisfactory resolution. The use of mixed buffers of this type has been described by Oulevey and Heitefuss[7], but their aim was to obtain a better separation of basic amino acids in a one-column system than could be achieved by use of a pure lithium buffer[10].

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In the two-column system, sodium buffers are generally still used for the elution of basic amino acids from the short column, as no better separation is achieved by lithium buffers. The use of potassium buffers has been described for elution of amines[11], where they were found to shorten the elution time compared with sodium buffers.

We also describe a method using potassium buffers for the elution of basic amino acids and related compounds from the short column.

MATERIAL AND METHODS

Equipment

A Beckman (Palo Alto, CA, U.S.A.) Model 121 amino acid analyzer equipped with an Autolab computing integrator was used. Two columns were employed, one 69 × 0.9 cm with 55 cm Beckman AA-15 resin for the acidic and neutral amino acids, and the other 28 × 0.9 cm with 16 cm Beckman PA-35 resin for the basic amino acids. A Hetofrig cooler was used for cooling the water in the column jackets.

Sample preparation

A synthetic mixture was prepared from amino acids obtained from Sigma (St. Louis, MO, U.S.A.), Merck (Darmstadt, G.F.R.), BDH (Poole, Great Britain) or Hoffmann-La Roche (Basle, Switzerland). The amino acids were dissolved in sodium citrate buffer. The amides glutamine and asparagine were renewed regularly. The plant amino acids were extracted according to Cook and Bieleski[12].

Reagents

The ninhydrin reagent was prepared from 100 g of ninhydrin, 4 l methyl Cellusolve, 1.5 l of 4 *M* sodium acetate buffer, pH 5.5, and 15 ml of 15% titanous chloride for reduction of the ninhydrin[13].

Three buffers were used on the long column and two on the short column for elution of the amino acids. Their compositions are listed in Tables I and II.

TABLE I
COMPOSITION OF BUFFERS FOR ELUTION OF ACIDIC AND NEUTRAL AMINO ACIDS FROM THE LONG COLUMN
The final adjustment of pH was made with HCl or LiOH–NaOH (3 mole/2 mole).

	Buffer		
	A	*B*	*C*
pH	3.00	3.30	4.15
Lithium concentration (*M*)	0.12	0.12	0.12
Sodium concentration (*M*)	0.08	0.08	0.08
Lithium citrate tetrahydrate (g/l)	11.28	11.28	11.28
Sodium citrate dihydrate (g/l)	7.84	7.84	7.84
Thiodiglycol (ml/l)	2.0	2.0	2.0
Caprylic acid (ml/l)	0.1	0.1	0.1
Concentrated HCl (ml/l)	14.0	12.5	9.5

TABLE II
COMPOSITION OF BUFFERS FOR ELUTION OF BASIC AMINO ACIDS FROM THE SHORT COLUMN

	Buffer	
	D	*E*
pH*	3.70	4.15
Potassium concentration (*M*)	0.30	0.40
Potassium citrate monohydrate (g/l)	21.63	21.63
Potassium chloride (g/l)	7.50	15.00
Caprylic acid (ml/l)	0.1	0.1
Concentrated HCl (ml/l)	11.0	9.5
Benzyl alcohol (ml/l)**	10.0	5.0

* Final adjustment made with HCl or KOH.
** Added after adjustment of pH.

For regeneration of the resins, 0.2 *M* lithium hydroxide–sodium hydroxide (Li^+–Na^+ = 3:2) and 0.2 *M* KOH were used for the long and short columns respectively.

Procedure

The flow-rate for the buffers was 70 ml/h and for the ninhydrin reagent 35 ml/h.

After sample injection, the amino acids on the long column were eluted with buffers A, B and C for 57, 90 and 98 min respectively. The two buffer changes were into effect between glutamine and glutamic acid and between cystine and methionine. The resin was regenerated with base for 18 min and equilibrated with buffer A for 44 min.

On the short column the amino acids were eluted with buffers D and E for 122 and 90 min with the buffer change between lysine and tryptophan. The resin was regenerated with base for 10 min and equilibrated with buffer D for 36 min.

The column temperature was lowered to 30°C by a 6-min fast cool step just before the injection of the sample on the long column. It was then maintained for 99 min before being raised to 45°C for the rest of the analysis.

RESULTS

The elution pattern of the first amino acids from the long column with the selected lithium–sodium ratio (0.12:0.08) is shown in Fig. 1, together with the effect of variation in this ratio at the chosen buffer pH and column temperature. This ratio gave a complete separation of the amides from each other and from the other amino acids. Glutamic acid was eluted after the amides and not between them as with the pure lithium buffer, which also gave a complete separation of the amino acids. When sodium formed a large part or all of the cations, poor or no separation of aspartic acid from threonine and of the amides was obtained.

The broad space between glutamic acid and glutamine achieved with the mixed buffer was utilized by placing the first buffer shift there.

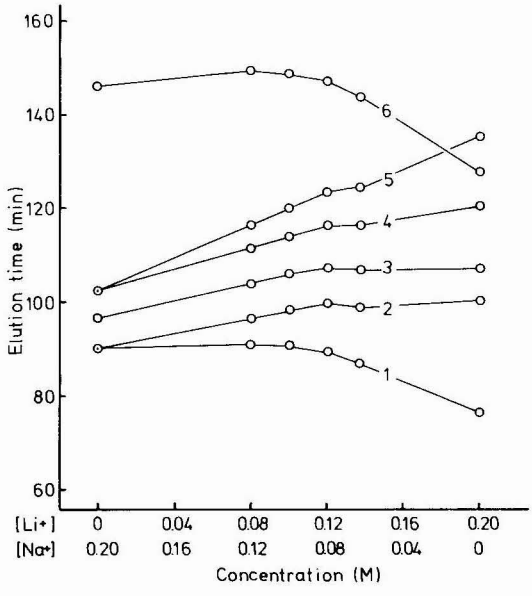

Fig. 1. Effect of the lithium–sodium ratio on the elution behaviour of the first common amino acids from the long column at pH 3.0; column temperature 30°C. Amino acids: 1 = Asp; 2 = Thr; 3 = Ser; 4 = Asn; 5 = Gln; 6 = Glu.

The column back pressure rose from 300 p.s.i. for a pure sodium buffer to 350 p.s.i. at the selection ratio and 450 p.s.i. for a pure lithium buffer.

A pH of 3.00 was found to be optimum for buffer A. Lowering the pH to 2.78 resulted in a much increased elution time and a partial overlapping of aspartic acid and threonine, while raising it to 3.24 resulted in coelution of the amides.

With the mixed buffers B and C the elution order of the common protein amino acids was as obtained for pure sodium or lithium buffers[2]. Unfortunately, α-aminoadipic acid and citrulline were not separated from the glycine–alanine couple and hydroxyproline was eluted together with aspartic acid, but these amino acids are normally present only in negligible amounts compared to the common protein amino acids.

The separation of basic amino acids and related compounds with the potassium buffer (D) is shown in Fig. 2, together with the effects of variation of the pH (a) and of the concentrations of potassium (b) and benzyl alcohol (c). A good separation of the named compounds was achieved except for tyrosine from phenylalanine, which, however, were separated on the long column. Tryptophan and arginine, which were eluted with buffer E, do not appear in the figure.

The optimum values for the parameters of buffer D were a pH of 3.70, a potassium concentration of 0.30 *M* and a benzyl alcohol concentration of 10 ml/l, with a reasonable flow-rate.

While most of the compounds are very sensitive to changes of pH in the tested range, ammonia and ethanolamine are unaffected. A decrease of pH to 3.6 gave a better separation of most of the compounds, but β-alanine came closer to ethanolamine. At pH 4.0 some amino acids are eluted together.

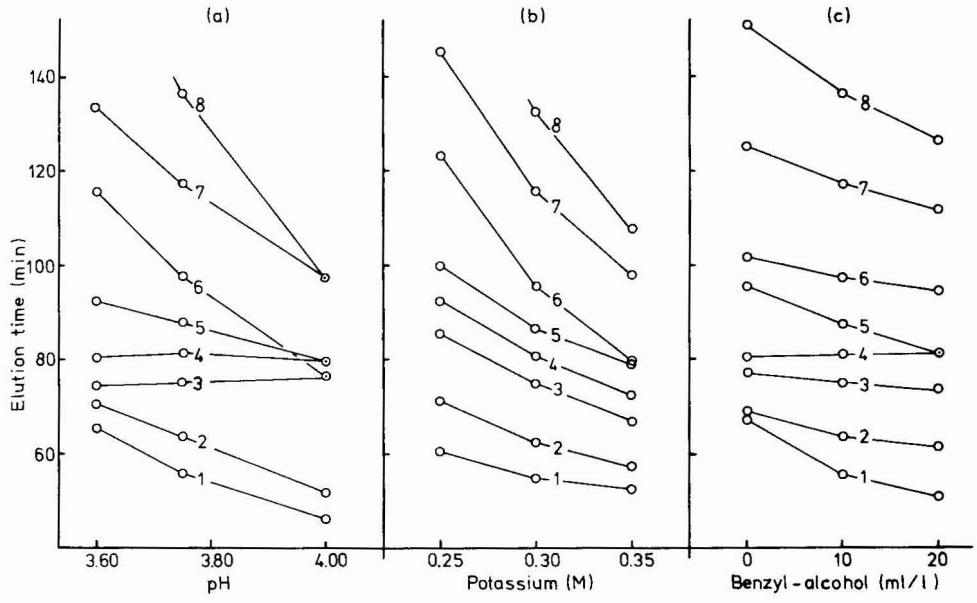

Fig. 2. Effect of the pH (a) and concentrations of potassium (b) and benzyl alcohol (c) on the elution behaviour of various amino compounds from the short column. Temperature 45°C; pH 3.75; potassium concentration 0.30 *M*; benzyl alcohol concentration 10 ml/l. Amino compounds: 1 = Tyr + Phe; 2 = β-Ala; 3 = ethanolamine; 4 = NH_3; 5 = γ-aminobutyric acid; 6 = Orn; 7 = His; 8 = Lys.

The potassium concentration had only a small effect on the elution pattern. With 0.25 *M* K^+ a better separation was achieved than with 0.30 *M* K^+, but at the expense of increased elution time and peak broadening. With 0.35 *M* K^+, ornithine and γ-aminobutyric acid were eluted together.

Addition of benzyl alcohol favoured the separation of β-alanine from tyrosine + phenylalanine because of a specific decrease in the elution time of the aromatic amino acid, especially of tryptophan, which appears late in the chromatogram. Also γ-aminobutyric acid was particularly affected and was eluted together with ammonia at 20 ml of benzyl alcohol per litre of buffer.

Changes in the column temperature had only small specific effects on the elution pattern.

A comparison of the sodium and potassium buffers was undertaken at the values of the buffer parameters found to give the best results with the potassium buffer (Table III). Most of the compounds are eluted faster with potassium than with sodium, except for the aromatic amino acids, tyrosine + phenylalanine and tryptophan.

Running of plant extracts prepared from grass and clover gave clear chromatograms with a good separation of the amino acids; some unidentified peaks appeared, especially before aspartic acid.

TABLE III
EFFECT OF TWO CATION SPECIES ON THE ELUTION TIMES OF VARIOUS AMINO COMPOUNDS FROM THE SHORT COLUMN
Buffer parameters: 0.2 *M* Na^+ or K^+; pH 3.75; 10 ml/l benzyl alcohol. Column temperature: 45°C.

Compounds	*Elution times (min)*	
	With K^+	*With* Na^+
Tyrosine + phenylalanine	55.5	51.5
β-Alanine	63.5	67.9
Ethanolamine	75.0	100.7
Ammonia	81.1	110.7
γ-Aminobutyric acid	87.4	100.7
Ornithine	97.6	137.0
Histidine	117.4	174.6
Lysine	136.2	195.1
Tryptophan	181.6	156.5

DISCUSSION

The mixed lithium–sodium buffer made it possible to separate the amides from each other and from the other amino acids and a lower pressure was obtained than with a pure lithium buffer. A higher proportion of lithium would have improved the separation of the first amino acids, but the column back pressure would have increased and there would have been less space between glutamine and glutamic acid for the buffer shift.

The specific effect of the cation species on the dicarboxylic amino acids is remarkable in that they, contrary to the other amino acids, are eluted faster with lithium than with sodium. Also α-aminoadipic acid is eluted sooner in relation to the other amino acids with lithium buffers[2]. A disadvantage of the mixed buffers is the more complicated preparation.

The effect of pH on the elution time of the amino compounds from the short column is dependent on whether their p*K* values lie within the applied range of pH. However, at the resin surface the pH is 1–2 units lower than in the buffer[14], which explains the stronger effect of pH on ornithine, lysine and histidine with p*K* values of 1.9, 2.2 and 1.8 respectively for their carboxylic group[15] than on β-alanine and γ-aminobutyric acid with p*K* values of 3.6 and 4.0 respectively. The small effect of pH on the elution of tyrosine and phenylalanine, which have p*K* values of about 2.2, is possibly due to a greater binding by non-electrostatic forces. Ammonia and ethanolamine have p*K* values too far outside the actual range of pH to be measurably influenced.

Benzyl alcohol probably acts as a competitor with respect to the adsorption by non-electrostatic forces and therefore exerts its strongest influence on the aromatic amino acids, which made it possible to separate β-alanine from tyrosine + phenylalanine. No effect was observed on ammonia and ethanolamine, which are mostly bound by electrostatic forces.

The faster elution of most of the compounds with potassium than with sodium is undoubtedly due to the smaller radius of the hydrated potassium ion. However, the aromatic amino acids exhibit opposite behaviour, with the advantage that the broad

peak of tryptophan is placed in the great space after lysine when potassium is used and not among the closely spaced amino compounds as with sodium. Tryptophan is anomalous in another respect in that it shows an increased elution time with increasing sodium concentration[16,17].

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REVERSED-PHASE THIN-LAYER CHROMATOGRAPHY OF DIACYLGLYCEROLS AS THEIR LABILE DIMETHYLBORATE ESTERS

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SUMMARY

In order to obtain a diacylglycerol mixture for reversed-phase chromatography, a triacylglycerol sample from native vegetable oils and having a pre-determined fatty acid composition was used. This sample, containing equimolar amounts of palmitic, stearic, oleic, linoleic and linolenic acids, was subjected to glycerolysis, and silica gel column chromatography of the products yielded a diacylglycerol mixture composed of 20 ± 3 mole-% of each of the above fatty acid residues. The mixture was separated into individual fractions by reversed-phase chromatography on plates with a permanent inert support layer using a methanol–trimethylborate–*n*-tetradecane system. The mixture was shown to consist of seven fractions with equivalent lipophilicities of 24–36, and the distribution of the residues of the individual fatty acids among these fractions is close to random.

INTRODUCTION

Although 1,2-*sn*-diacylglycerols (1,2-*sn*-DAGs) form the basis of the structure of natural glycerolipids[1], little is known about the composition and structure of the DAG moieties of these lipids, mainly because methods for the analysis of DAGs have been insufficiently developed[2]. In our laboratory, work is being conducted on devising a method for the chromatographic separation of natural DAG mixtures, formed by the hydrolysis of polar glycerolipids, into individual species. These mixtures, however, are not suitable for developing this method because in most instances they are not readily available and have limitations with respect to their fatty acid composition[1]. Therefore, we used an artificial mixture of pre-determined composition obtained by glycerolysis. This mixture serves as an adequate substitute for a natural mixture because the pattern of DAG mobility in liquid chromatography usually does not depend on the isomeric composition of the DAG species[3].

In addition to the preparation of a DAG mixture and the development of a chromatographic method to separate it into individual fractions, an aim of this work was also the preliminary identification of the isolated fractions as regards their equivalent lipophilicity[4], which is necessary for the possible future application of these

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fractions as standards in the investigation of DAGs of natural origin. Finally, in connection with the possibility of such an application of a standard DAG mixture, it was of interest to establish the rules governing the distribution of fatty acid residues among the individual DAG fractions in the mixture.

EXPERIMENTAL

Materials

Triacylglycerols (TAGs) from cacao butter recrystallized from a 10% (w/v) solution in *n*-hexane and TAGs from poppy seed[5] and linseed oils[6] were purified on an alumina column[7]. As shown by thin-layer chromatography (TLC), TAGs eluted from the column were devoid of more polar components. Tripalmitoyl- and tristearoylglycerols (C grade; California Foundation for Biochemical Research, Los Angeles, CA, U.S.A.) were used without further purification. The amount of the major component in the samples of these monoacid TAGs was assumed to be 100%. The TAG sample for glycerolysis was obtained by mixing TAGs from cacao butter (2.63 g), linseed oil (3.73 g) and poppy seed oil (2.17 g), and by adding tristearoyl- (0.84 g) and tripalmitoylglycerol (0.63 g).

TAG glycerolysis and isolation of DAG mixture

To 10.00 g (11.5 mmole) of the TAG sample 1.30 g (14.1 mmole) of anhydrous glycerol and 11 mg (0.3 mmole) of sodium hydroxide as a catalyst for glycerolysis were added. The mixture was incubated in a continuous stream of argon at low pressure (*ca.* 10 mmHg) at 200°C for 4 h, dissolved in diethyl ether, washed with 20% sodium chloride solution until neutral, dried and evaporated to dryness *in vacuo*. As a result, 10.78 g of an acylglycerol sample was obtained. To determine its composition both 100 μg of the sample and 100 μg of neutral lipid standards[8] were spotted at the starting points of a Silufol plate (Kavalier, Sklárny, Czechoslovakia). After TLC separation for 15 min using *n*-hexane–diethyl ether–acetic acid (50:50:1), the spots of the lipids were rendered visible with molybdophosphoric acid[8].

A column (42 × 2 cm I.D.) containing 60 g of Woelm silica gel for adsorption chromatography instead of the Davidson Grade 923 silica gel used earlier[9] was applied to isolate the DAG mixture (1.11 g). To determine the purity of the latter, a 100-μg aliquot was separated by TLC (see above); for comparison, 0.5 μg of TAGs were applied at one of the starting points. The colour intensity of individual chromatographic zones was evaluated visually.

DAG samples originating from cacao butter and poppy seed and linseed oils were prepared and evaluated in a similar manner. The fatty acid compositions of all acylglycerol samples and mixtures were determined by gas–liquid chromatography[10].

TLC plates and mobile phase

DAGs were separated on home-made glass plates designated as "plates with a permanent support layer". To prepare such plates, 20 g of Kieselguhr (Chromaton N; Chemapol, Prague, Czechoslovakia, particle size 0.100–0.125 mm) was silanized with dimethyldichlorosilane[11], mixed with 60 g of powdered glass[8] and suspended in 90 ml of water, and the slurry obtained was applied on to 20 × 10 cm plates using an SSG-1 automatic layer applicator (VEB Technisches Glas, Ilmenau, G.D.R.); the

applicator clearance was 0.5 μm. After the layer had been dried in air at 20°C the plates were heated at 675°C for 20 min[8]. These plates could be used for separation an unlimited number of times, because they can be purified from organic impurities by heating them in mixture of concentrated sulphuric and nitric acids and developing the support layer in methanol.

As the mobile phase for DAG separation we first used a mixture of methanol and water (95:5). Subsequently, a mixture of 475 g (14.8 mole) of absolute methanol and 111 g (1.8 mole) of reagent-grade boric acid was refluxed for 3 h, cooled and kept with 154 g (1.1 mole) of sodium sulphate for 45 h to remove water formed during esterification of the acid. The supernatant liquid was decanted and fractionally distilled, the methanol–trimethylborate (TMB) azeotrope, b.p. 54.6–55.0°C, n_D^{20} 1.3478, being collected[12]. A 1:9 mixture of this azeotrope with absolute methanol, plus a small excess of pure *n*-tetradecane, was used for chromatography.

Chromatographic separation and semi-quantitative evaluation of DAG mixture

To apply the stationary phase the plates were submerged in a 10% (v/v) benzene solution of tetradecane. Separate DAG samples and a mixture of them were applied on the starting line as a band (65 × 2 mm) with a GSG-1 automatic sample applicator (VEB Technisches Glass) fixed on the carriage of the SSG-1 instrument, or by a syringe as a series of single spots. At the end portions of the band (1–2 mm long) where the carriage changed the direction of its shuttle motion, a greater amount of DAG than in the middle portion of the band could be deposited, which subsequently resulted in significant distortion of the chromatographic zones. To avoid this, these portions of the layer were covered with paper strips when the sample had been applied.

DAGs (200 μg on the starting line or 20–50 μg as single spots) were separated for 2.5 h in a chamber for ascending TLC (Type OE-304; Labor, Budapest, Hungary) at 23 ± 0.5°C. *n*-Tetradecane was removed from the plates in a vacuum drying oven (Type LP-402; Labor) for 45 min at 140°C. After development of the spots[8], the chromatogram was photographed and a positive picture was obtained from the negative. The picture was scanned under transmitted light on a home-made densitometer. The peak areas on the densitogram were determined gravimetrically. When calculating the hR_F (R_F × 100) values the maximum of the peak on the densitogram was assumed to be the centre of the corresponding zone.

RESULTS

Composition of the mixed TAG sample for glycerolysis

A DAG mixture in which molar contents of individual DAG species are as similar to each other as possible is desirable as a chromatographic standard. As can be calculated[13], this condition is most readily met when each fatty acid in the starting TAG sample is present in equal molar concentrations. On the other hand, as this standard was designed primarily for investigating common plant lipids, it had to be similar to them in its qualitative fatty acid composition. It is known that the individual aliphatic acids present in these lipids are very numerous, but in almost all instances residues of the five major acids palmitic, stearic, oleic, linoleic and linolenic acid are present. Therefore, we decided to limit ourselves to the preparation of a DAG mixture containing only these five acids.

Thus, the optimal starting material for glycerolysis in our work would be an equimolar mixture containing 20% of each of the monoacid TAGs of the five major acids. However, reagent-grade samples of such TAGs, especially unsaturated ones, are often unavailable, so we decided to prepare a TAG sample from the total TAGs of common, readily available vegetable oils; these TAGs could be easily isolated from the oils by adsorption chromatography. Cacao butter rich in saturated and oleic acids, poppy seed oil with predominantly linoleic acid and linseed oil as a source of linolenic acid were used (Table I). It can be seen that the oleic content in the cacao butter sample was about 35% and in two other oils it was close to 20%, a pre-determined value for the whole sample. Therefore, to attain this value in the sample it was necessary to include additionally small amounts of the simple saturated TAGs tripalmitoyl- and tristearoylglycerol.

TABLE I
FATTY ACID COMPOSITION OF ACYLGLYCEROLS (MOLE-%)

Fatty acid	*TAGs from the fat of*			*TAG sample before glycerolysis*	*Acylglycerol sample*	*DAG mixture*
	Cacao	*Poppy seed*	*Linseed*			
Palmitic	27.1	9.3	5.7	17.2	17.4	19.7
Stearic	35.8	4.4	3.8	17.6	18.9	18.4
Oleic	34.9	16.3	19.6	21.6	20.6	19.8
Linoleic	2.2	70.0	11.6	23.3	23.5	22.9
Linolenic	0.0	0.0	59.3	20.3	19.6	19.2

For direct preparation of the sample (total weight 10.00 g) it was necessary to determine the weights (g) of TAGs of cacao butter, poppy seed and linseed oils, and of tripalmitoyl- and tristearoylglycerol (X, Y, Z, V and W, respectively), which would ensure the pre-determined fatty acid composition. X g of cacao TAGs contain

$$X' = X/\bar{M}_x \tag{1}$$

mole of TAGs, where $\bar{M}_x = \sum_i [(a_i)_x M_i] \cdot 10^{-2}$ = average molecular weight of cacao butter TAGs, $(a_i)_x$ (mole-%) = content of the ith acid (Table I) and M_i = molecular weight of the monoacid TAG of the ith acid[6]; values of $Y' = Y/\bar{M}_y, \ldots, W' = W/\bar{M}_w$ (mole) are expressed similarly.

Using these values, one can calculate, as a dimensionless quantity, the molar proportion of cacao TAGs in the sample:

$$x = X'/(X' + \ldots + W') \tag{2}$$

The values of y, z, v and w are expressed similarly ($x + y + z + v + w = 1$).

The pre-determined content of stearic acid (20%) in the TAG sample to be prepared is made up of the amounts of this acid in TAGs from each of the oils:

$$35.8x + 4.4y + 3.8z + 100w = 20 \tag{3}$$

Similarly, for oleic and linoleic acids:

$$34.9x + 16.3y + 19.6z = 20 \quad (4)$$

and

$$2.2x + 70.0y + 11.6z = 20 \quad (5)$$

Preliminary experiments showed that the content of palmitic acid in the DAG mixture obtained by glycerolysis is higher and that of linolenic acid is lower than the contents in the starting TAG sample. Therefore, we decided to mix TAGs so that the calculated amounts of these acids in the starting sample were 18 and 22%, respectively:

$$27.1x + 9.3y + 5.7z + 100v = 18 \quad (6)$$

and

$$59.3z = 22 \quad (7)$$

From eqns. 3–7, $x = 0.263$, $y = 0.216$, $z = 0.371$, $v = 0.068$ and $w = 0.082$. By substituting the values of $x, \ldots, w$ into eqns. 1 and 2 for X and X', and into similar equations for $Y, \ldots, W$ and $Y', \ldots, W'$, and by combining this equation system with the expression $X + Y + Z + V + W = 10$ g, one can solve it and obtain the TAG weights: $X = 2.63$ g, $Y = 2.17$ g, $Z = 3.73$ g, $V = 0.63$ g and $W = 0.84$ g.

The fatty acid composition of the starting TAG sample is shown in Table I; it is close to the pre-determined value.

Preparation of the DAG mixture

As suggested by the preliminary data available[3], individual DAG species, on reversed-phase chromatography and on liquid chromatography in the presence of complex-forming agents, are not separated into positional isomers [1,2(2,3)- and 1,3-diacyl-*sn*-glycerols] of the same fatty acid composition[1]. Hence, any reaction that gives rise to all of these isomers or to only some of them generally can be used to prepare model DAGs for reversed-phase chromatography, because individual DAG species of the mixture will have the same mobility as corresponding 1,2-diacyl-*sn*-glycerols of natural origin.

In this work we obtained the DAG mixture by glycerolysis, which is largely free from limitations inherent in other methods of DAG synthesis on the micro-scale[13,14]. In an anhydrous medium the glycerolysis reaction, *i.e.*, transesterification of TAGs with glycerol in the presence of a catalyst[15–17], involves TAG deacylation and continuous acyl migration between TAGs, DAGs, monoacylglycerols (MAGs) and glycerol; as a result, dynamic equilibrium between all of these compounds is established. As can be seen from the Table I, there is little, if any, difference between the overall acylglycerol sample formed by TAG glycerolysis and the original TAGs as regards their fatty acid composition. The TLC data (not shown) suggest that DAGs comprise almost half of the sample, whereas free fatty acids which would interfere with the isolation of the DAG mixture are virtually absent.

The isolation itself was carried out using a silica gel column, and the degree of purity of the mixture obtained was determined by TLC. The DAG content in the final mixture was >99.5%, and the yield was about 50%. The fatty acid composition of DAGs is shown in the last column in Table I. It can be seen that the actual concentrations of individual acids are around 20 ± 3 mole-%.

Reversed-phase TLC of the DAG mixture

DAGs were separated chromatographically in a reversed-phase system[18]. Instead of paper chromatography[19], TLC was used because of its greater separation efficiency. However, under the usual conditions the solid support containing a hydrophobic stationary phase is poorly retained on the surface of a glass plate and is easily washed off by the mobile phase[20]. Therefore, plates with a permanent inert support (Kieselguhr) layer were used for TLC. These plates were prepared by the method developed previously for adsorption plates[8].

As aqueous water-soluble organic solvent–hydrophobic phase systems have been used successfully for separating free fatty acids[4], TAGs[15], MAGs[21] and other neutral lipids, we carried out experiments on DAG fractionation in these systems. The hydrophobic phases tested included higher hydrocarbons, long-chain esters and ethers and aliphatic methyl ketones, and the hydrophilic components used included acetic acid, methanol and acetone. In no instance, however, was a satisfactory DAG separation achieved. As an example, Fig. 1 shows the results of the analysis of a standard DAG mixture in the aqueous methanol–*n*-tetradecane system.

We then considered the adsorption chromatography of hydroxy lipids. In some of these studies, to increase the selectivity and quality of the separation of hydroxy fatty acids[22], and positional isomers of MAGs and DAGs[23,24], other workers used adsorbents impregnated with boric acid, which is known[25] to form labile complexes with free hydroxyl groups. It could be assumed that in a reversed-phase system the

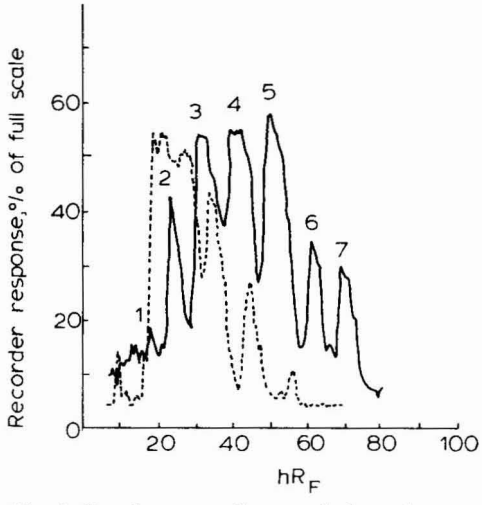


Fig. 1. Densitograms of reversed-phase chromatograms of diacylglycerols. Mobile phases: methanol–water (95:5) saturated with *n*-tetradecane (broken line) and methanol–trimethylborate (92.7:7.3) saturated with *n*-tetradecane (solid line).

addition of this acid or its derivatives to the mobile phase would also result in an improvement of the chromatographic parameters, in a similar manner to the results with Ag^+ ions, which initially were applied only for the adsorption chromatography of neutral lipids[2] but were later used in their reversed-phase chromatography[26].

To verify this assumption, experiments on DAG separation were conducted in a system containing methanolic solutions of either boric acid (5%) or an equimolar amount of TMB as the mobile phase. Under these conditions, a highly selective and efficient separation of DAGs into seven fractions was achieved, and when the TMB system devoid of free water was used the chromatographic mobility of separate DAG fractions, especially the fully saturated ones, increased even further (Fig. 1). Therefore, only the latter system was used in subsequent work. The results of the semi-quantitative densitometric determination of the concentration of each of these fractions in the DAG mixture are presented in Table II.

TABLE II
DENSITOMETRIC ANALYSIS OF THE CONTENT OF SEPARATE FRACTIONS IN A MIXTURE OF DIACYLGLYCEROLS

*Fraction No.**	hR_F	*Content (wt.-%)*
1	20	2– 6
2	28	9–11
3	36	14–21
4	44	23–27
5	52	21–28
6	60	8–14
7	68	7–12

* DAG peak numbers in Fig. 1.

Tentative identification of DAG fractions

For the tentative identification of the isolated DAG fractions (Fig. 1), it is first necessary to establish the distribution pattern of DAGs in a reversed-phase chromatographic system of moderate efficiency. It can be assumed that DAGs, being neutral lipids, do not differ in this respect from other lipids of this kind, such as TAGs and fatty acids. The distribution of these lipids is known to be determined by their theoretical equivalent lipophilicity, $L_1 = m - 2e$, where m and e are the number of carbon atoms and the number of double bonds, respectively, in the aliphatic chains of a given molecule[27]. As a result, neutral lipids belonging to the same class and with identical L_1 values also have identical hR_F values in a reversed-phase system.

Identification of the fractions could also be based on the assumption that the composition of DAGs produced in the reaction of the glycerolysis of oils is "random", *i.e.*, it reflects the random distribution of fatty acid residues among the individual DAG species. In order to establish the extent to which the data in Fig. 1 and Table II correspond to this composition, it is necessary, proceeding from the number (n) and the concentration (a, b, ... %) of the individual fatty acid residues in the DAG mixture (Table I), to calculate the random number of DAG species (N) and the random concentration of each species (A_1, A_2, ... %), and then to compare the calculated results with the experimental ones (Table II).

To derive the equations for the calculation of N, and also of A_1, A_2, ..., we modified the available probability equations for TAGs[15]. It is clear that N is made up of the numbers of monoacid (n) and diacid [$n(n-1)/2$] species of DAGs, *i.e.*, $N = n(n+1)/2$; in our mixture, where $n = 5$, $N = 15$. In a similar way, for each monoacid DAG species $A_1 = a^2/100$, and for the diacid species $A_2 = 2\,ab/100$; the factor of 2 has been introduced into this equation to take into account both positional isomers of DAGs. On considering the random composition of DAG species calculated from the qualitative composition and the quantitative content of fatty acid residues in their mixture (Table I) and presented in Table III, one can see that in the number of fractions of definite lipophilicity the DAG mixture isolated here (Table II) conforms to the random composition, and the quantitative content of each of these fractions is also sufficiently close to random (± 1–10%).

TABLE III
RANDOM SPECIES COMPOSITION OF A MIXTURE OF DIACYLGLYCEROLS

Species and fractions of DAGs	*m**	*e**	L_1	*Random content of DAGs (wt.-%)***	
				Species	*Fractions*
Distearoylglycerol	36	0	36	3.6	3.6
Stearoylpalmitoylglycerol	34	0	34	6.9	14.5
Stearoyloleoylglycerol	36	1		7.6	
Dipalmitoylglycerol	32	0	32	3.3	23.6
Palmitoyloleoylglycerol	34	1		7.4	
Stearoyllinoleoylglycerol	36	2		8.8	
Dioleoylglycerol	36	2		4.1	
Palmitoyllinoleoylglycerol	34	2	30	8.5	25.2
Stearoyllinolenoylglycerol	36	3		7.3	
Oleoyllinoleoylglycerol	36	3		9.4	
Palmitoyllinolenoylglycerol	34	3	28	7.1	20.3
Oleoyllinolenoylglycerol	36	4		7.8	
Dilinoleoylglycerol	36	4		5.4	
Linoleoyllinolenoylglycerol	36	5	26	9.0	9.0
Dilinolenoylglycerol	36	6	24	3.8	3.8

* m and e are the number of carbon atoms and number of double bonds, respectively, in the aliphatic chains of DAG molecules.

** Random contents of DAGs are expressed in weight-% and calculated in accordance with the theory of random distribution from the fatty acid composition of the model DAG mixture (Table I).

For an even more accurate identification of separate fractions in the standard DAG mixture by the L_1 value, the composition of this mixture was compared with those of DAG samples isolated from the products of glycerolysis of cacao butter and poppy seed and linseed oils, which differ greatly in their fatty acid compositions (Table I). To carry out this comparison each of the three samples, and also the DAG mixture, were applied on the same TLC plate, separated, detected with molybdophos-

phoric acid and quantified by densitometry. The found and random contents of separate DAG fractions in the samples are shown in Table IV. The number of fractions found in each sample (four for cacao and poppyseed, six for linseed) and in the mixture (seven) depends on the number of individual acids in the corresponding original oil (four, four and five, respectively; Table I). As in the case of a standard DAG mixture, the number of separate fractions conforms in all instances to the random composition and their content is close to that calculated using the equations of random distribution.

TABLE IV
FOUND AND RANDOM CONTENTS OF SEPARATE FRACTIONS IN DIACYLGLYCEROL SAMPLES OBTAINED BY GLYCEROLYSIS OF CACAO BUTTER AND POPPYSEED AND LINSEED OILS (WT.-%)
Found values, obtained by densitometry; random values, calculated in accordance with the theory of random distribution from the fatty acid composition of the corresponding fats (Table I) expressed in weight-%.

hR_F	*Cacao*		*Poppyseed*		*Linseed*		*Identification of fractions by* L_1
	Found	*Random*	*Found*	*Random*	*Found*	*Random*	
20	1– 3	9.0	–	0.2	–	0.2	36
28	16–33	39.6	2– 3	2.3	0– 3	2.2	34
36	52–55	45.8	14–18	12.6	9–18	6.7	32
44	14–25	5.4	34–37	35.3	13–21	11.0	30
52	–	0.2	45–47	49.6	23–27	29.9	28
60	–	0.0	–	0.0	13–18	13.4	26
68	–	0.0	–	0.0	27–42	36.6	24

As can be seen from the chromatogram in Fig. 2, this mixture has almost the same hR_F values of the corresponding DAG fractions as the DAG samples. With decreasing lipophilicity of the fractions these values increase, and relationship between hR_F and L_1 in Table IV is linear: $hR_F = 164 - 4\,L_1$. This equation, reflecting the polarity of the mobile phase in a TMB chromatographic system, can be used in the L_1 range 16–41. The final results of the identification of the fractions, characterized by certain hR_F values, by their lipophilicities are given in Table IV.

Thus, the standard mixture of DAGs includes seven separate fractions having hR_F values of 20, 28, 36, 44, 52, 60 and 68 and L_1 values of 36, 34, 32, 30, 28, 26 and 24, respectively. The distribution of the residues of individual fatty acids among these fractions appears to be close to random.

DISCUSSION

The glycerolysis conditions specified under Experimental appear to be optimal because the DAG yield was higher than in most instances reported by other workers[28,29]. At lower temperatures transesterification would be markedly slower[16], whereas higher temperatures would decrease the yield of reaction products owing to their thermal degradation, polymerization and removal of glycerol by distillation[30]. At the chosen temperature the highest glycerolysis rate is attained on adding 0.1–0.2% of sodium hydroxide to the reaction mixture[31]; sodium hydroxide is also ad-

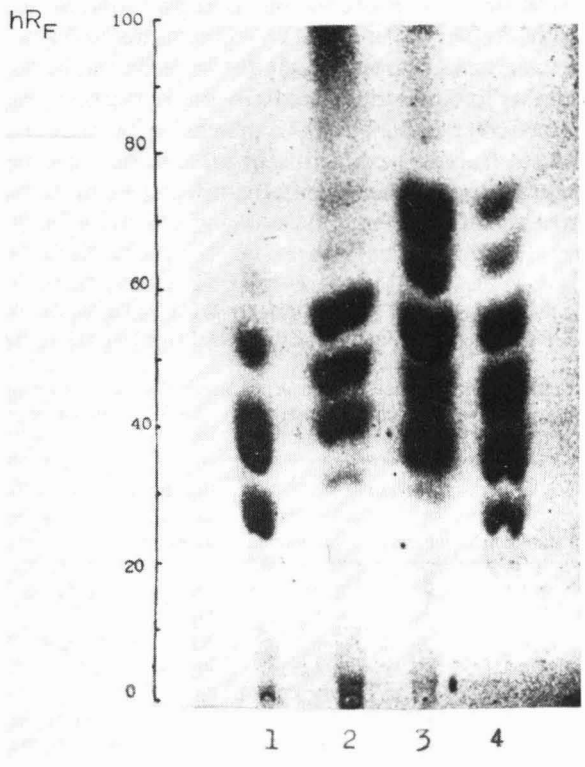


Fig. 2. TLC of diacylglycerols in the reversed-phase system. Mobile phase: methanol–trimethylborate saturated with *n*-tetradecane (Fig. 1). 1–3 = DAG samples (20 μg each) from cacao butter and poppy seed and linseed oils, respectively; 4 = DAG mixture (50 μg).

vantageous as a catalyst because it lacks marked fatty acid specificity in the transesterification reaction[16]. The highest DAG content in the products of this reaction (>40 mole-%) was obtained when the TAG:glycerol molar ratio was 7–17[17], compared with 7.7 in our sample. We also reproduced the gas-phase conditions of glycerolysis, *i.e.*, a slight vacuum[31] and an inert atmosphere[16].

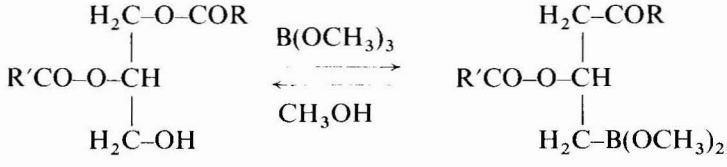
If natural lipids having qualitative and quantitative fatty acid compositions other than those used here were to be employed as starting materials for this reaction, then, using the scheme for TAG sample preparation suggested above, it would be possible to obtain acylglycerol mixtures with a virtually unlimited range of fatty acid compositions. Such mixtures could be useful as standards for reversed-phase chromatography, and also for creating various desirable fatty compositions for biochemical, chemical and technological lipid studies.

Our use of reversed-phase chromatography for the fractionation of DAG mixtures is uncommon, because in recent years DAGs have been separated into individual components almost exclusively by the GLC of their acetyl, trimethylsilyl and other derivatives, and by adsorption TLC of these derivatives with silver ions[1]. However, these methods involve a number of difficulties, especially the need to synthesize the derivatives. The yields of these derivatives, especially when obtained on the micro-

scale, have usually not been cited, and the derivatives themselves often appear to be insufficiently stable[32]. Moreover, when performing such syntheses there is inevitably migration of acyl residues in DAGs[1]. Finally, the selectivity of the chromatographic fractionation of DAG derivatives by the methods used in the past is limited: in GLC effective separations and quantitative determinations can be accomplished only for compounds with different chain lengths, and in Ag^+ TLC only for derivatives with different numbers of double bonds[2].

Reversed-phase chromatography has been used for a long time to determine the species composition of many classes of neutral lipids, primarily TAGs[2], as it is characterized by mild fractionation conditions and a high efficiency and selectivity of separation. At the same time, this method has been applied to the analysis of DAG composition only in the early period of the development of reversed-phase chromatography[19,33] and, therefore, it was of interest to test it again for this purpose, taking into account recent achievements in this type of chromatography.

Our results show that the reversed-phase fractionation of DAGs became possible only when TMB was used, which resulted in the substitution of the free hydroxyl group due to the formation of dimethylborate (DMB) esters of DAGs in this system:



Previously DMB esters of MAGs were identified as the products of the reaction between acetonides of monoacylglycerols and boric acid[25]. DMB esters of DAGs were formed upon detritylation of trityl-DAG in the presence of boric acid[32] and upon adsorption chromatographic separation of positional isomers of DAGs in a solvent system containing TMB[24]; in both instances the formation of DMB esters effectively inhibited acyl migration in DAGs.

An important advantage of DMB esters is that in the presence of water they are very rapidly hydrolysed, yielding initial acylglycerols[25], whereas acetates and other DAG derivatives (see above) cannot be used for recovering native DAGs. Another advantage is that the chromatographic fractionation of DMB esters is not accompanied by the additional separation of the zones of individual DAGs into positional isomers (Fig. 1), although these isomers are known to be present in the DAG mixture under study because it was obtained by glycerolysis of TAGs. Hence the use of these esters makes it possible to perform with equal success the analysis of any DAG mixture, irrespective of its isomeric composition, without preliminary isolation of individual isomers.

The separation of the mixture of the DAGs as their DMB esters by reversed-phase chromatography showed that the concentrations of the separate fractions in this mixture are close to those calculated according to the random distribution pattern. Thus, such a distribution of fatty acyls is established not only in TAGs formed by glycerolysis[13] but also in other products of this reaction, especially in DAGs. This pattern of DAG composition is also supported by the similarity between DAG mixtures and the original TAGs with respect to their fatty acid composition; if DAG formation proceeded in accordance with a selective mechanism this similarity would not exist. It was previously shown that under the chosen conditions of glycerolysis the reaction does not reach the equilibrium state and, as a result, the DAG concentration

(mole-% of the total acylglycerols) is considerably higher than the random concentration[34]. Consequently, during glycerolysis of TAGs a random distribution of acyl residues first appears inside the DAG class and only later between the other classes of acylglycerols.

This kind of distribution in the artificial DAG mixture will serve as an important advantage in the use of this mixture as a chromatographic standard because it will ensure the comparison of 1,2-*sn*-DAGs of natural origin with the widest range of DAG species, *i.e.*, with all the DAG species possible for a given fatty acid composition in a mixture.

CONCLUSION

The proposed method of reversed-phase chromatography of the DMB derivatives of DAGs in the methanol–trimethylborate–*n*-tetradecane system appears to be suitable for the analysis not only of model DAG mixtures but also of DAG samples obtained by the enzymatic hydrolysis of polar glycerolipids, and the standard mixture can be used as a standard for the chromatographic identification of DAGs with L_1 = 24–36 in terms of their lipophilicities and for the three DAG species with L_1 = 24,26 and 36 according to their structures.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR QUANTITATION OF FREE ACIDS, MONO-, DI- AND TRIGLYCERIDES USING AN INFRARED DETECTOR

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SUMMARY

Methyl ester, mono-, di- and triglyceride classes were separated and quantitated by high-performance liquid chromatography. Baseline separations were achieved on an amino-, cyano-bonded Partisil column with a gradient from hexane–chloroform (60:65) to hexane–chloroform–acetonitrile (25:65:35). The eluate was monitored by an infrared detector set at 5.72 μm, and peak areas were determined by computer integration. Methyl 9,10-dihydroxystearate was used as an internal standard. The total run time and recycling time was 35 min. A precision of 10% or better was obtained for all components, even at the 1% (w/w) level.

INTRODUCTION

Separation and quantitation of mixtures of fatty acids and mono-, di- and triglycerides are often desired in natural products, emulsifier additives and food chemistry. Thin-layer (TLC)[1–4] and column[5,6] chromatographic methods separate lipid classes, but are time consuming and require additional techniques for quantitation of the fractions. Chromarods[7,8] and gas chromatography (GC)[9–11] have been used to quantitate certain components, but they do not permit isolation of material for further study. High-performance liquid chromatography (HPLC) with ultraviolet detection at 213 nm is not quantitative for mixtures containing a variety of unsaturated components[12].

To date, a dual-gradient HPLC system utilizing the transport flame ionization or "moving wire" detector has shown the most success in quantitation of lipid classes[13]. We have now developed a system of single-gradient HPLC utilizing infrared (IR) detection that separates and quantitates acyl-containing lipid classes.

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TABLE I
COMPOSITION AND ANALYSIS OF STANDARD MIXTURES

Mixture No.	*No. of trials*	*Standard**		*Methyl palmitate*				*Tripalmitin*			
		mmoles/ml	*mg/ml*	*mmoles/ml*	*mg/ml*	M_y/M_s**	$\bar{x}(A_y/A_s)$***	*mmoles/ml*	*mg/ml*	M_y/M_s	$\bar{x}(A_y/A_s)$
1	5	0.0303	10.01	0.0298	8.04	0.984	1.096	0.0125	10.06	0.413	1.303
2	6	0.0243	8.01	0.0112	3.01	0.461	0.494	0.0075	6.03	0.309	1.130
3	4	0.0152	5.01	0.0223	6.02	1.467	1.460	0.0037	3.02	0.243	0.840
4	5	0.0091	3.00	0.0372	10.03	4.088	3.919	0.0121	9.76	1.330	4.043
5	5	0.0303	10.01	0.186	5.02	0.614	0.608	0.0061	4.88	0.201	0.628

Mixture No.	*No. of trials*	*Standard**		*1,3-Dipalmitin*				*Monopalmitin*			
		mmoles/ml	*mg/ml*	*mmoles/ml*	*mg/ml*	M_y/M_s	$\bar{x}(A_y/A_s)$	*mmoles/ml*	*mg/ml*	M_y/M_s	$\bar{x}(A_y/A_s)$
1	5	0.0303	10.01	0.0177	10.06	0.584	1.203	0.0305	10.05	1.007	0.965
2	6	0.0243	8.01	0.0177	10.06	0.728	1.504	0.0152	5.03	0.626	0.574
3	4	0.0152	5.01	0.0142	8.05	0.934	1.820	0.0304	10.05	2.000	1.685
4	5	0.0091	3.00	0.0089	5.03	0.978	1.851	0.0292	9.64	3.209	2.545
5	5	0.0303	10.01	0.0025	1.41	0.083	0.145	0.0146	4.82	0.482	0.383

* Methyl 9,10-dihydroxystearate.
** M_y = moles of component y; M_s = moles of standard.
*** A_y = peak area of component y; A_s = peak area of standard.

EXPERIMENTAL*

Methyl palmitate, tripalmitin, dipalmitin and monopalmitin of purity greater than 99% were obtained from Nu-Chek-Prep (Elysian, MN, U.S.A.). A product sold as "methyl 10,11-dihydroxystearate" was obtained from K & K Labs. (Plainview, NY, U.S.A.). Four grams of this product were placed on a dry column (21 cm × 2 cm I.D.) of Hi-Flosil and eluted with a litre of chloroform. The eluate was evaporated to dryness, and the residue was recrystallized from 600 ml of cold (−10°C) diethyl ether. Mass spectrometry proved that the purified ester was actually methyl 9,10-dihydroxystearate.

Five standard mixtures (Table I) were prepared by dissolving known amounts of each of the following in chloroform: methyl palmitate, tripalmitin, dipalmitin, methyl 9,10-dihydroxystearate and monopalmitin. The concentration of each component in each solution is given in Table I. In addition, the ratio of the moles of each component (M_y) to moles internal standard (M_s) is given for comparison with the analytical findings. The ratio of the peak area (A_y) of each component to the area of the internal standard (A_s) was calculated for each trial, and the averages of the trials, $\bar{x}$ (A_y/A_s), are given in Table I.

Two other standards were also prepared. Crude soybean oil was lipolyzed and recovered[14], and the resulting mixture was treated with diazomethane[15] to convert the free acids into methyl esters. Methyl 9,10-dihydroxystearate (65.2 mg) was added to 28.2 mg of this mixture in 0.7 ml of chloroform. In a second sample, 282.8 mg of commercial shortening and 2.07 mg of methyl 9,10-dihydroxystearate were dissolved in 0.25 ml of chloroform.

HPLC was accomplished on a column (25 cm × 4.6 mm) of Partisil PXS 10/25 PAC (Whatman, Clifton, NJ, U.S.A.), using gradient elution. Solvent A was hexane–chloroform (60:65) and solvent B was acetonitrile–hexane–chloroform (35:25:65). A linear gradient of these two solvents went from 2 to 95% B in 20 min at 2 ml/min. The chromatographic system included two M-6000A pumps, a Model 660 solvent programmer (all from Waters Assoc., Milford, MA, U.S.A.) and an IR detector for liquid chromatography (DuPont, Wilmington, DE, U.S.A.) set at 5.72 μm and 0.1 A attenuation.

Typically, samples of 0.5 to 1.5 mg were injected, although 55 mg of the commercial shortening were injected because the components of interest represented only 1 to 4% (w/w) of the total mixture. A typical run time was 35 min, with 6–7 min needed to recycle before the next injection.

A laboratory-wide computer system[16] was used to determine peak areas. Each HPLC run was stored in the computer and then displayed on an interactive graphics terminal. The cross-hairs of the terminal were used to mark the beginning, end and baseline location for each peak. The area above the baseline was calculated by the computer, and an area report was printed. This rapid interactive method was required because a solvent front and the triglyceride peak eluted simultaneously. In laboratories not equipped with a manually controlled computer integration system, similar

* The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

control over beginning, end and baseline of each peak can be obtained by using the "cut and weigh" method.

RESULTS AND DISCUSSION

In the early stages of work, it was found that free acids tailed and overlapped the diglycerides. Esterification of the free acids in the sample before analysis eliminated these difficulties and allowed quantitation of the free acids (as methyl esters). Inclusion of an internal standard permitted independent quantitation of individual components. Methyl 9,10-dihydroxystearate was selected as internal standard because it is readily available, reasonably inexpensive, and gives a peak that is well resolved from others in the chromatogram.

Most available solvents (both Spectro and ACS grades) contain traces of materials that absorb at 5.72 μm. Hexane and acetonitrile had absorbances of similar intensities at 5.72 μm, but chloroform was one of the few solvents that had virtually no absorbance in that region. Careful balancing of the amounts of hexane, acetonitrile and chloroform in solvents A and B was necessary to produce a baseline that had relatively little drift as the program progressed. A typical baseline (Fig. 1a) was generated from a trial in which only chloroform, the solvent used in sample preparation, was injected. The chloroform produced a negative peak *ca.* 1.4 min after injection.

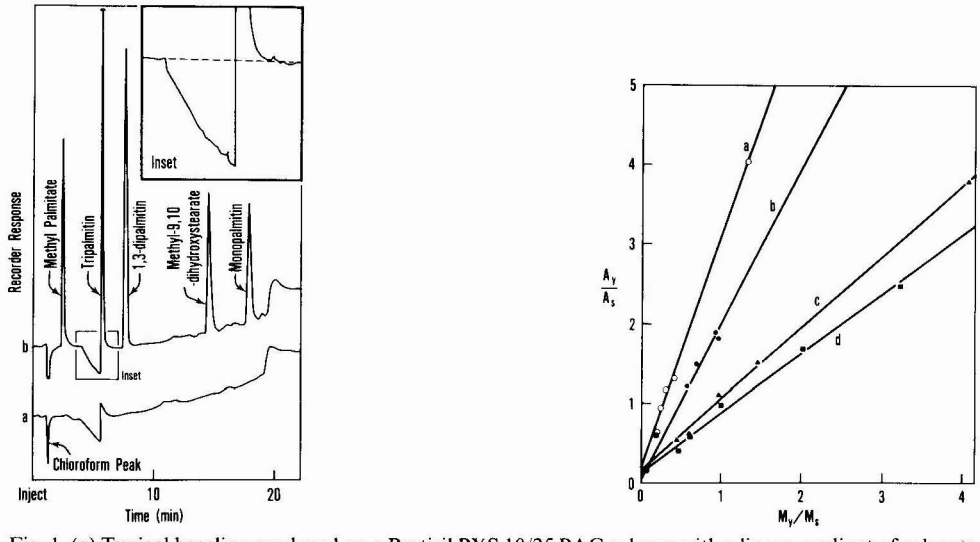


Fig. 1. (a) Typical baseline produced on a Partisil PXS 10/25 PAC column with a linear gradient of solvents A [hexane–chloroform (60:65)] and B [acetonitrile–hexane–chloroform (35:25:65)] from 2 to 95% in 20 min at 2 ml/min monitored at 5.72 μm; chloroform (5 μl) was injected at the start of the program. (b) HPLC chromatogram of standard mixture 1 (methyl palmitate, tripalmitin, dipalmitin, methyl 9,10-dihydroxystearate and monopalmitin) under the conditions in (a). Inset: Integration of triglyceride peak. Dotted line shows baseline used.

Fig. 2. Plots of area of component/area of standard methyl 9,10-dihydroxystearate (A_y/A_s) *versus* moles of component/moles of standard (M_y/M_s) for methyl palmitate, tripalmitin, dipalmitin, and monopalmitin based on the mixtures listed in Table I. (a), tripalmitin (slope, 2.9); (b), dipalmitin (slope, 1.9); (c), methyl palmitate (slope, 0.89); (d), monopalmitin (slope, 0.75).

Other irregularities in the baseline resulted from the gradient programming[17]. The baseline dipped from 4 to 6 min, then rose sharply. To integrate the triglyceride peak that eluted with this baseline shift, the baseline following the peak was extended horizontally through to the beginning of the peak (see inset in Fig. 1). This technique provided a consistent method of determining peak area and gave results in good agreement with the known amounts of sample. At *ca.* 20 min, a second baseline shift occurred. Interference between this shift and the monoglyceride peak was eliminated by running the system at 95 % B for 10 min before recycling for the next run. Since re-equilibration of the column by switching directly back to initial conditions took only 6 to 7 min, a "reverse" gradient[17] was not necessary. When the system was idle for several hours or overnight, a "warm-up" run through the entire sequence optimized conditions for later chromatography.

Five standard mixtures of methyl palmitate, tripalmitin, dipalmitin, methyl 9,10-dihydroxystearate and monopalmitin (Table I and Fig. 1) were each analyzed four to six times. Since detection is based on absorbance due to carbonyl groups, molar response factors of 3 for triglycerides, 2 for diglycerides and 1 for monoglycerides and for methyl esters would be predicted. In Table I, the values of $\bar{x}(A_y/A_s)$ are approximately three times the values of M_y/M_s for tripalmitin, twice the values of M_y/M_s for dipalmitin and the same as the values of M_y/M_s for methyl palmitate and monopalmitin. To determine the actual response factors, values of $\bar{x}(A_y/A_s)$ for each component were plotted against (M_y/M_s) (Fig. 2). The slopes of the lines were: tripalmitin, 2.9 (σ 0.07); dipalmitin, 1.9 (σ 0.07); methyl palmitate, 0.89 (σ 0.05); and monopalmitin, 0.75 (σ 0.07). These values were used as response factors (f_y) in calculation of moles/g of subsequent samples using the equation

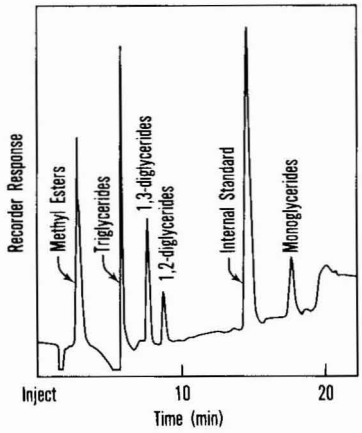
$$\frac{M_y}{\text{g sample}} = \frac{A_y M_s}{A_s f_y w}$$

where w is the weight of the sample.

However, the predicted response factors (number of carbonyl groups per molecule) are close enough to the experimental values that they could be used to obtain approximate values of M_y/g sample. The overall relative standard deviation for the method was 7.1 %. By running each sample in duplicate, 95 % confidence limits (C.L.) of ± 10.0 % were obtained ($2s/r$ = C.L.).

In the study of natural products, it is often desirable to quantitate classes of compounds such as glyceride fractions composed of species having several molecular weights. Results in molar units from IR detection of carbonyl functionalities are more convenient for evaluating these mixtures than the weight percentage obtained from flame ionization and "moving wire" detectors. For example, in the lipolysis procedure used to determine the glyceride structure of oils, the extent of hydrolysis is often calculated from molar ratios[9]. If results are originally in weight percent, an average molecular weight for each class must be determined before the results can be converted to moles.

To demonstrate an application of the HPLC–IR method to natural systems, a soybean oil lipolysis mixture[9,14] was prepared and analyzed (Fig. 3). Mean values (mmole/g) of four trials were: methyl esters, 4.49 (σ 0.07); triglycerides, 0.62 (σ 0.05); 1,3-diglycerides, 0.802 (σ 0.003); 1,2-diglycerides, 0.35 (σ 0.02); and monoglycerides 1.40 (σ 0.08). The overall relative standard deviation was 4.9 %.



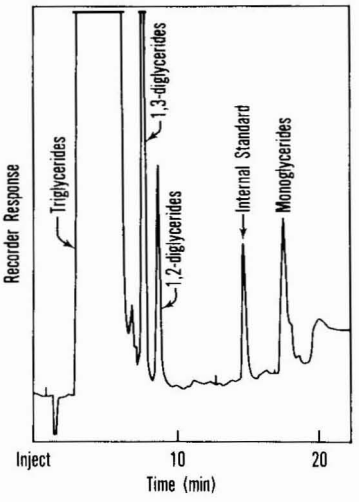


Fig. 3. HPLC chromatogram of soybean oil lipolysis mixture. Conditions as for Fig. 1.

Fig. 4. HPLC chromatogram of a commercial shortening. Conditions as for Fig. 1.

A further advantage of this method is that components present in small amounts (*ca.* 1%) can be measured to within $\pm 0.1\%$ by increasing the sample size. Mono- and diglycerides present at 1–4% (w/w) levels in a commercial shortening were quantitated by adding an appropriate amount of standard and injecting a large sample (55 mg) on to the column (Fig. 4). Mean values (mmole/g) in six trials were: 1,3-diglycerides, 0.0387 ($\sigma \pm 0.0025$); 1,2-diglycerides, 0.0195 ($\sigma \pm 0.0015$); and monoglycerides, 0.0527 ($\sigma \pm 0.0008$). The overall relative standard deviation was 5.9%. By adjusting the internal standard and sample sizes, even smaller amounts of monoglycerides could probably be quantitated with similar precision.

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RAPID MICRO-METHOD FOR THE MEASUREMENT OF PARACETAMOL IN BLOOD PLASMA OR SERUM USING GAS–LIQUID CHROMATOGRAPHY WITH FLAME-IONISATION DETECTION

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SUMMARY

A simple method for the measurement of plasma paracetamol concentrations using a novel extraction/acetylation procedure prior to gas–liquid chromatographic analysis has been developed. The sample (100 μl) is vortex-mixed for 30 sec with 0.067 mole/l phosphate buffer, pH 7.4 (50 μl), internal standard solution (N-butyryl-*p*-aminophenol (200 mg/l) in chloroform) (50 μl) and "acetylation reagent" (acetic anhydride–N-methylimidazole (catalyst)–chloroform, 5:1:30) (20 μl). After centrifugation at 9950 *g* for 3 min, a portion (5 μl) of the resulting extract is analysed on a 1.5 m × 4 mm I.D. glass column packed with 3% (w/w) C_{87} hydrocarbon (Apolane-87) on Chromosorb W HP, 100–120 mesh, maintained at 235°C. A specimen together with a quality control sample can be analysed, in duplicate, within 20 min. The limit of accurate measurement of the assay is 10 mg/l, and few potential sources of interference have been identified. The method has advantages of speed and reproducibility over other gas–liquid chromatographic procedures and, in addition, of selectivity over spectrophotometric techniques. The procedure provides a useful alternative to liquid chromatographic methods for emergency paracetamol measurements.

INTRODUCTION

The rapid measurement of plasma paracetamol (acetaminophen) concentrations can be important in the diagnosis and management of acute paracetamol poisoning[1], especially with respect to cases presenting more than 12 h after ingestion of the drug[2]. The available methods have been reviewed by Wiener[3]. Chromatographic techniques have advantages of selectivity and small sample requirement when compared with spectrophotometric methods, and a rapid high-performance liquid chromatographic assay has been described recently[4].

The method presented here for the measurement of plasma paracetamol concentrations involves the formation of the acetyl derivatives of paracetamol and of the internal standard, N-butyryl-*p*-aminophenol, prior to gas–liquid chromatographic (GLC) analysis[5]. However, studies with N-methylimidazole (*ca.* 360 times more efficient than pyridine in the catalysis of the acetylation of isopropanol[6]) have prompt-

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ed the simplification of the procedure to the extent that the extraction and derivatisation can be accomplished in a single step.

EXPERIMENTAL

Materials and reagents

Paracetamol and N-butyryl-*p*-aminophenol were obtained from Sigma London (Gillingham, Great Britain) and from Winthrop (Surbiton, Great Britain), respectively. The latter compound was used as a 200 mg/l solution in chloroform (analytical-reagent grade) which was stable for at least 3 months if stored at 4°C in the absence of light. The "acetylating reagent" was a mixture of acetic anhydride (analytical-reagent grade)–N-methylimidazole (Aldrich, Gillingham, Great Britain)–chloroform (5:1:30), and was prepared daily. Sodium dihydrogen orthophosphate, disodium hydrogen orthophosphate (both analytical reagent grade) buffer, 0.067 mole/l, pH 7.4 ("phosphate buffer") was prepared in distilled water.

Gas–liquid chromatography

A Pye 104 gas chromatograph equipped with dual flame-ionisation detectors was used, and integration of peak areas was performed using a Hewlett-Packard 3352 data system. The column oven temperature was 235°C and the detector oven temperature was 300°C; injection port heaters were not employed. The column, a 1.5 m × 4 mm I.D. glass tube, was silanised by treatment with 2% dichlorodimethylsilane in toluene for 8 h, rinsed with methanol and dried at 100°C prior to packing with 3% (w/w) C_{87} hydrocarbon (Apolane-87) on Chromosorb W HP, 100–120 mesh, purchased ready-prepared from Pierce and Warriner (Chester, Great Britain). The carrier-gas (nitrogen) flow-rate was 40 ml/min, and the hydrogen and oxygen inlet pressures were 15 and 10 p.s.i., respectively.

The chromatography on this system of a derivatised extract of an aqueous solution of paracetamol to which N-butyryl-*p*-aminophenol had been added is illustrated in Fig. 1. The retention times on this system of some compounds extracted under the conditions of the assay, measured relative to the internal standard, are given in Table I.

Sample preparation

Plasma or serum (100 µl), internal standard solution (50 µl), phosphate buffer (50 µl) and acetylating reagent (20 µl) were added to a small test (Dreyer) tube (Poulten, Selfe and Lee, Wickford, Great Britain). Hamilton gas-tight luer-fitting glass syringes (2.5, 2.5 and 1.0 ml, respectively) fitted with Hamilton repeating mechanisms and stainless steel needles were used in the addition of these latter volumes. The contents of the tube were vortex-mixed for 30 sec and the tube was centrifuged at 9950 *g* for 3 min in an Eppendorf centrifuge 5412 (Anderman, East Molesey, Great Britain) which was modified to accept Dreyer tubes by drilling-out the 0.4-ml test-tube centrifuge adapters. Subsequently, a portion (3–5 µl) of the chloroform layer was taken and injected onto the gas chromatographic column using a syringe fitted with an 11.5-cm needle. The whole procedure was carried out at room temperature, normally 22°C.

Duplicate sample analyses were performed and the mean results taken.

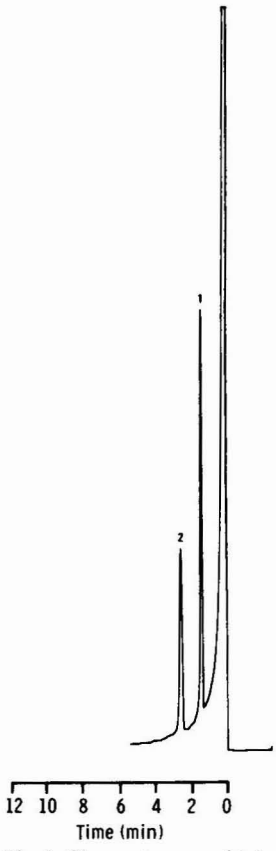

Fig. 1. Chromatogram obtained on analysis of an extract of an aqueous solution containing paracetamol (200 mg/l) on the C_{87} hydrocarbon system; 3-μl injection. The initial N-butyryl-*p*-aminophenol concentration was 200 m/l. Peaks: 1 = acetylated paracetamol; 2 = acetylated N-butyryl-*p*-aminophenol. See text for chromatographic conditions.

Instrument calibration

Solutions containing paracetamol at concentrations of 20, 50, 100, 200, 300 and 400 mg/l were prepared in distilled water by dilution of a 1 g/l paracetamol solution in methanol. These solutions were stable for at least 3 months if stored at 4°C in the absence of light. On analysis of these solutions, the ratio of the peak area of acetylated paracetamol to that of the internal standard was linear (Fig. 2). The calibration gradient normally obtained was 0.008 l/mg, with an intercept of −0.086.

RESULTS AND DISCUSSION

Choice of extraction conditions

Although some procedures involving the GLC analysis of underivatised paracetamol have been described, the majority of published methods incorporate a derivatisation step prior to chromatographic assay[3]. However, these derivatisations were not only time-consuming in themselves, but also required prior solvent extrac-

TABLE I
RETENTION TIMES OF ACETYLATED PARACETAMOL, N-BUTYRYL-*p*-AMINOPHENOL AND SOME OTHER COMPOUNDS ON TWO-COLUMN SYSTEMS
See text for chromatographic conditions.

Compound	*Retention time (relative to N-butyryl-p-aminophenol)*	
	C_{87} hydrocarbon system	*SP-2250 system*
Ethosuximide	0.19	0.15
Barbitone	0.32	0.27
Methyprylone	0.36	0.32
Ibuprofen	0.40	(0.21 and 0.29)
Allobarbitone	0.41	0.35
Aprobarbitone	0.45	0.37
Meprobamate	0.47	0.71
Butobarbitone	0.48	0.39
Allylbarbitone	0.50	0.38
Methsuximide	0.50	0.37
Amylobarbitone	0.54	0.43
Phenacetin	0.55	0.51
Paracetamol (acetylated)	0.59	0.68
Chlorpropamide	0.60	0.37
Pentobarbitone	0.63	0.48
Quinalbarbitone	0.68	0.55
Ethotoin	0.76	0.87
Methoin	0.77	0.76
"Glutethimide metabolite"	0.84	1.58
Phenylethylmalondiamide	0.85	1.19
Brallobarbitone	0.87	0.85
Hexobarbitone	0.91	0.79
Caffeine	0.93	0.92
Glutethimide	0.96	0.82
Lignocaine	1.00	0.73
Phenazone	1.00	0.95
N-Butyryl-*p*-aminophenol (acetylated)	1.00	1.00
Amidopyrine	1.08	1.00
Thiopentone	1.09	0.68
Diphenhydramine	1.12	0.67
Propylphenazone	1.18	1.07
"Glutethimide metabolite"	1.20	1.00
Phenobarbitone	1.22	1.26
Cyclobarbitone	1.29	1.17
Orphenadrine	1.36	0.83
Heptabarbitone	1.74	1.57
Cyclizine	2.21	1.25

tion and concentration steps, rendering the procedures too lengthy for emergency use The introduction[6] of N-methylimidazole as both catalyst and proton-accepting base in place of pyridine in the catalysis of acetylation reactions prompted the present work. N-Methylimidazole, a liquid at room temperature, is not only a more efficient catalyst than pyridine but is also widely available, non-toxic and miscible with water

Although it was thought that a large excess of acetic anhydride would be required if the derivatisation of paracetamol was attempted in the presence of water

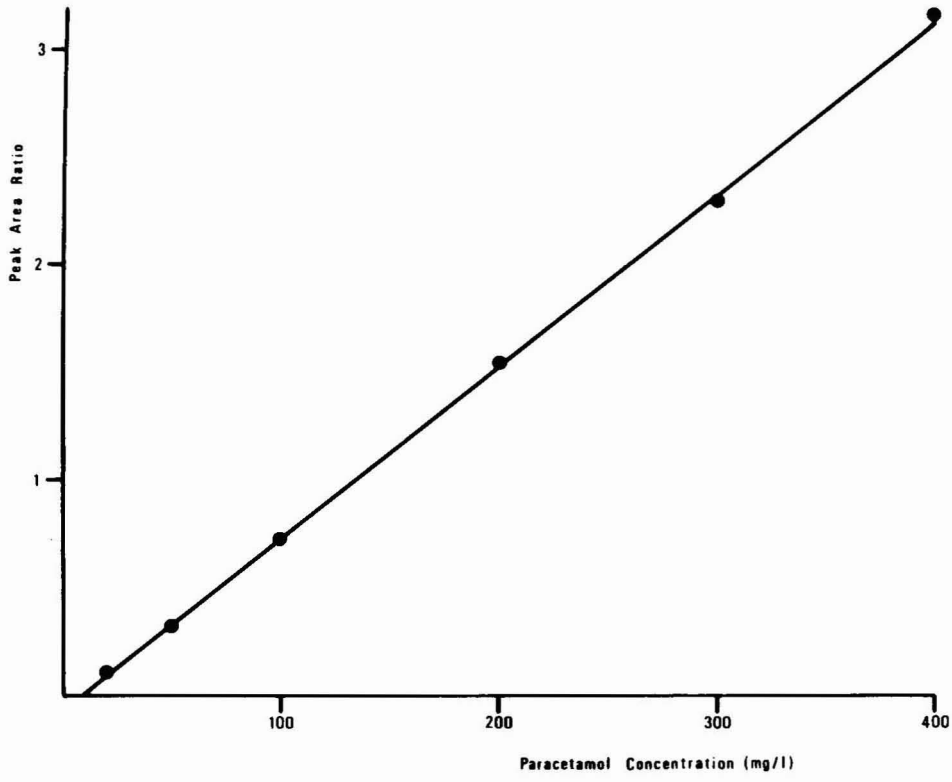

Fig. 2. Calibration graph of peak area ratio of acetylated paracetamol to internal standard against paracetamol concentration in the aqueous calibration standards.

the direct use of an acetic anhydride–catalyst–chloroform mixture has allowed the simple extraction procedure to be used. In practice, it was found that only relatively small amounts of both acetic anhydride and N-methylimidazole were required, and this had the advantage of restricting the size of the "solvent front" obtained on GLC analysis of the extract to that normally associated with the injection of chloroform alone (Fig. 1). Phosphate buffer was used in the assay in order to yield a final extraction mixture with a pH of *ca.* 6. On analysis of extracts of standard aqueous paracetamol solutions, both paracetamol and the internal standard gave rise to sharp, symmetrical peaks (Fig. 1), suggesting that the acetylated derivatives had been formed. Analysis of these standard solutions performed without the addition of acetic anhydride did not give rise to any peaks, whereas treatment of both paracetamol and N-butyryl-*p*-aminophenol with N-methylimidazole–acetic anhydride (2:1, v/v) for 10 min at room temperature gave rise to compounds with retention times identical with those obtained on the direct analysis of aqueous paracetamol solutions (Table I).

Recovery studies

Standard solutions containing paracetamol at concentrations of 50, 100 and 250 mg/l were prepared in heparinised bovine plasma by dilution of a 1 g/l methanolic

solution of the drug. The apparent recoveries of acetylated drug were 94.9 ± 1.1, 94.5 ± 1.3 and 95.8 ± 0.4 (S.E.M.)%, respectively (n = 10 at each concentration), when compared with the aqueous calibration solutions. (*N.B.* The latter solutions were preferred for routine use in view of their greater stability with respect to plasma standards[7].)

Sources of interference

No endogenous sources of interference have been observed. The chromatogram obtained on analysis of a specimen of drug-free human plasma is illustrated in Fig. 3, and that obtained using a specimen from a patient who had ingested paracetamol is shown in Fig. 4. Analyses undertaken without the addition of the internal

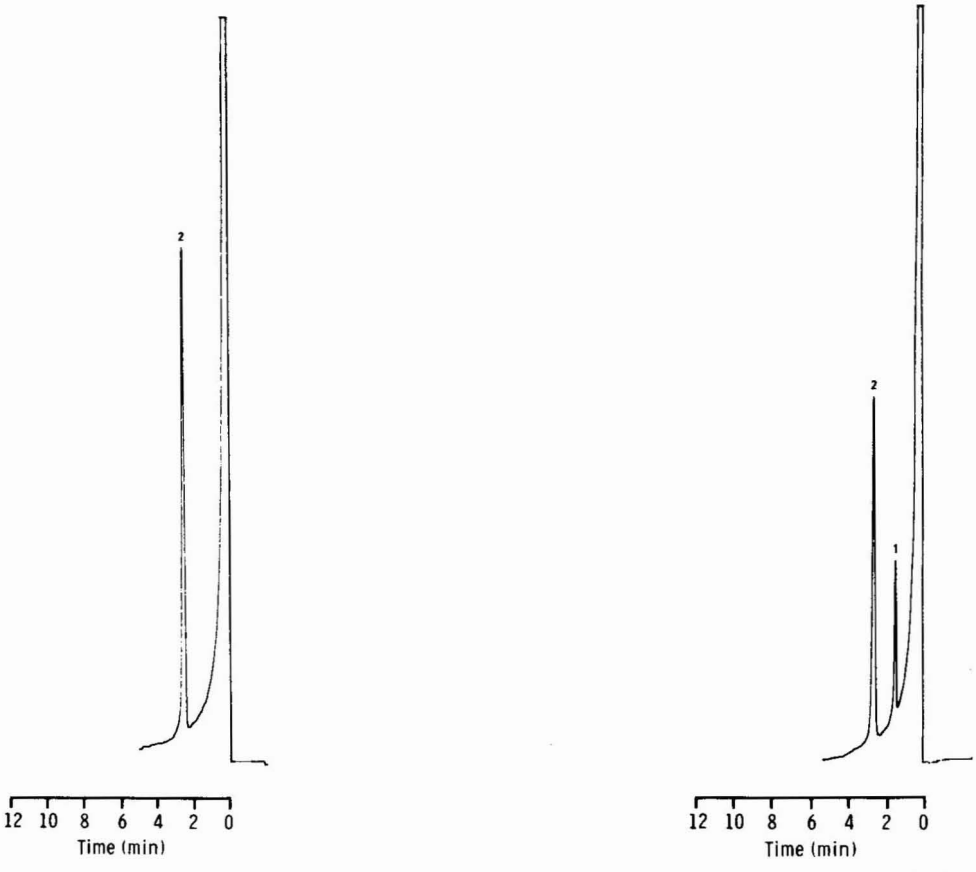

Fig. 3. Chromatogram obtained on analysis of an extract of drug-free human plasma on the C_{87} hydrocarbon system; 5-μl injection. The initial N-butyryl-*p*-aminophenol concentration was 200 mg/l, and peak 2 is acetylated N-butyryl-*p*-aminophenol.

Fig. 4. Chromatogram obtained on analysis of an extract of plasma from a patient who had ingested paracetamol on the C_{87} hydrocarbon system; 4-μl injection. The initial N-butyryl-*p*-aminophenol concentration was 200 mg/l, and the plasma paracetamol concentration was found to be 44 mg/l. Peaks: 1 = acetylated paracetamol; 2 = acetylated N-butyryl-*p*-aminophenol.

standard have not revealed the presence of endogenous compounds which could interfere with this latter substance. Analyses of specimens obtained *post mortem* have given analogous results.

Assessment of potential interference from other drugs or exogenous agents is complicated by the need to consider not only drugs directly extracted under the conditions of the assay but also additional compounds which could be acetylated/extracted. However, the range of compounds involved is probably no greater than with methods incorporating separate extraction and derivatisation steps[5]. A further consideration is that the concentrations of paracetamol attained following overdosage are relatively high (in our experience up to 1 g/l) when compared with most centrally-acting agents such as barbiturates, glutethimide or methaqualone[8], suggesting that only compounds potentially occurring at plasma concentrations of the order of 10 mg/l or greater need be considered. Finally, if the presence of interfering compounds is suspected, analysis of specimens without addition of the acetylating reagent but using pH 4.5 buffer[8] provides a simple means of differentiating between compounds extracted directly under the conditions of the assay and those, such as paracetamol and N-butyryl-*p*-aminophenol, which have to be acetylated prior to chromatographic analysis.

No interference from exogenous compounds has been observed during the routine operation of the assay over a nine-month period, during which time specimens from patients who had ingested or been treated with a wide variety of compounds were analysed. (*N.B.* Whenever possible, the identification of paracetamol in plasma specimens was confirmed by a colour test performed by addition of *o*-cresol–ammonia to a hydrolysed urine specimen[9].) In particular, interference from salicylate, diflunisal, ibuprofen and benorylate or from metabolites of paracetamol or chlormethiazole was not observed. Of the drugs which were extracted and chromatographed under the conditions of the assay (Table I), several (including phenacetin) present potential sources of interference. (*N.B.* Some additional compounds, such as phenytoin, primidone, carbamazepine, azapropazone, methaqualone and the benzodiazepines eluted at retention times greater than 1.80 relative to acetylated N-butytyl-*p*-aminophenol.) Phenacetin was resolved from paracetamol on a second relatively polar column system, 3% SP-2250 on Chromosorb W HP, 80–100 mesh (Chromatography Services, Hoylake, Great Britain). The retention times of the compounds under study on this second system are given in Table I, and the separation of acetylated paracetamol and N-butyryl-*p*-aminophenol is illustrated in Fig. 5. The chromatographic conditions employed for this second system were identical with those used for the C_{87} hydrocarbon system.

Interference from "cresol B.P." (a mixture of *o*-, *m*- and *p*-cresol) and "chlorocresol B.P." (4-chloro-3-methylphenol) did not occur. These compounds are used as preservatives in mucous heparin solutions and, as reported by Pitts[10], contamination from the solution containing "cresol B.P." especially can cause serious interference in the widely-used spectrophotometric paracetamol assay of Glynn and Kendal[11]. Contamination of this type is possible if a specimen is drawn initially into a heparinised blood–gas syringe, and may not be recognised unless the absorption spectrum of the final reaction product is examined. Similarly, potential interference from salicylate in this latter method, although not of great magnitude, necessitates either examination of the final absorption spectrum or simultaneous salicylate measurement[3]. As with all

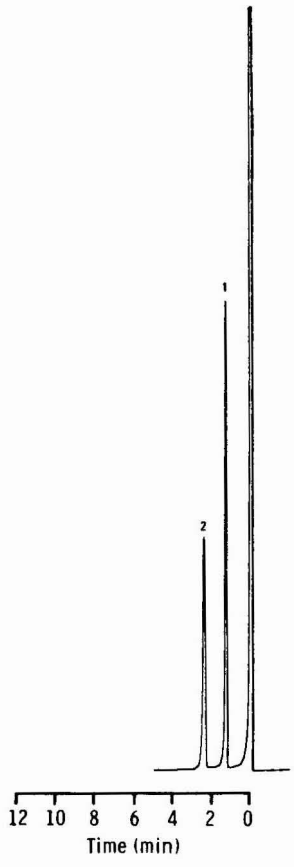

Fig. 5. Chromatogram obtained on analysis of an extract of an aqueous solution containing paracetamol (200 mg/l) on the SP-2250 system; 4-μl injection. The initial N-butyryl-*p*-aminophenol concentration was 200 mg/l. Peaks: 1 = acetylated paracetamol; 2 = acetylated N-butyryl-*p*-aminophenol. See text for chromatographic conditions.

spectrophotometric methods for plasma paracetamol assay, interference from this or other sources can lead to equivocal results, especially at relatively low plasma concentrations. This can be very important where the patient presents, or paracetamol overdose is suspected on clinical grounds, many hours after ingestion of the drug. In such cases, the diagnosis often rests upon the accurate measurement of the residual paracetamol plasma concentration.

Reproducibility

The intra-assay coefficients of variation (C.V.) obtained on replicate analysis of standard solutions of paracetamol prepared in expired blood-bank plasma[7] were 4.2% at 48 mg/l (n = 10) and 2.3% at 238 mg/l (n = 10).

Limit of sensitivity

The limit of accurate measurement of the assay was 10 mg/l; the intra-assay C.V. at this concentration was 9.2% (n = 7).

External quality control scheme

Paracetamol solutions prepared in expired blood-bank plasma[7] were analysed by the present method over a nine-month period. There was a good correlation between the results obtained (mean = 195 mg/l, range 50–476 mg/l) and the weighed-in paracetamol value (mean = 192 mg/l; range 48–492 mg/l) ($r = 0.99$; $n = 27$). Linear regression analysis using the weighed-in value as the independent variable revealed a gradient of 1.06 and an intercept on the y-axis of -7.9 mg/l.

Catalyst concentration, extraction time and extract stability

In order to investigate the effect of such variables as the concentration of N-methylimidazole on the apparent recovery of acetylated paracetamol and internal standard, a second non-derivatisable standard was incorporated in the procedure. The compound chosen, *n*-tetracosane (C_{24}) (Koch-Light, Colnbrook, Great Britain), eluted at a retention time of 1.42 on the SP-2250 system relative to acetylated N-butyryl-*p*-aminophenol, and was used as a 1 g/l solution in chloroform (analytical-reagent grade). The investigations were performed using standard solutions of paracetamol (100 mg/l) prepared in water or expired blood-blank plasma. The extractions were performed as described previously in triplicate, except that *n*-tetracosane solution (20 µl) was added to each tube prior to vortex mixing and, in the first instance, changes were made in the amount of N-methylimidazole added. Peak-height measurements of both acetylated paracetamol and N-butyryl-*p*-aminophenol were performed relative to the peak height of *n*-tetracosane.

Use of an acetylating reagent consisting solely of acetic anhydride–chloroform (1:6) with an aqueous paracetamol solution did give rise to acetylated paracetamol and N-butyryl-*p*-aminophenol (mean peak height ratios 0.39 and 0.28, respectively), but these ratios were less than those obtained using the normal acetylating reagent (mean ratios 0.50 and 0.40, respectively) and there was greater variation in the individual results. Re-mixing and subsequent analysis of the extracts performed without the addition of the catalyst did give an increased yield of the acetylated compounds (mean ratios 0.46 and 0.34, respectively), but no major change was observed following this sequence using the normal extracts (mean ratios 0.48 and 0.41). Addition of N-methylimidazole at twice the normal concentration in a further experiment did not give rise to an increased yield of acetylated products (mean ratios 0.50 and 0.41, respectively).

Information on the adequacy of the 30-sec mixing period was obtained from the experiment above where the results of the re-mixing and re-analysis of normal extracts were quoted. The stability of these extracts was shown by re-analysis of the extracts after 8 h at ambient temperature, both before and after re-mixing, which gave identical results (mean ratios 0.48 and 0.41, respectively) to those given above. Analyses of the plasma paracetamol solution gave analogous results to those obtained with the aqueous standard with respect to extraction time and extract stability, given the slightly lower relative recovery of paracetamol from plasma as compared with aqueous solutions.

CONCLUSIONS

The method described here has been found to be suitable for the measurement

of the plasma paracetamol concentrations attained after overdosage with this compound. N-Methylimidazole is preferable to pyridine as a catalyst in the derivatisation procedure since the former compound is widely available and is also non-toxic, and thus the procedure can be carried out in the open laboratory. Neither extract concentration nor prolonged heating is employed, and only 200 μl of specimen are required for a duplicate analysis which can be completed, together with the analysis of a quality control specimen, within 20 min. The procedure is relatively specific, and offers a useful alternative to liquid chromatographic procedures for emergency plasma paracetamol measurements.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF IDOXURIDINE AND RELATED SUBSTANCES ON CHEMICALLY BONDED OCTADECYLSILYL SILICA PACKINGS

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SUMMARY

The high-performance liquid chromatographic procedures for the assay of idoxuridine and the test for related substances described in the British Pharmacopoeia 1980 were investigated. It was found possible to optimize the composition of the eluent with respect to the elution time without loss of separation efficiency. Ten commercially available octadecylsilyl-bonded silica packings were investigated in order to find any suitable supports other than the one prescribed. The packings exhibited large differences in retention behaviour and selectivity. An impurity, which the test for related substances does not take into account, was found in some samples of idoxuridine and it has been isolated and identified as 5-bromo-2′-deoxyuridine.

INTRODUCTION

The British Pharmacopoeia 1980 (BP 80)[1] includes a monograph on idoxuridine, which describes the assay of the drug by high-performance liquid chromatography (HPLC). A test for related substances is also performed by HPLC, and the same procedures are mentioned in the monograph on idoxuridine eye-drops. The test for related substances is limited to the two degradation products 2′-deoxyuridine and 5-iodouracil, *cf.*, the main degradation routes of idoxuridine shown in Fig. 1.

The HPLC procedures for pharmacopoeial purposes involve the use of a prescribed eluent and support. Because some interfering peaks corresponding to unknowns were found in the chromatograms of some samples of this drug substance and in some preparations using a support of the prescribed nature, we have attempted

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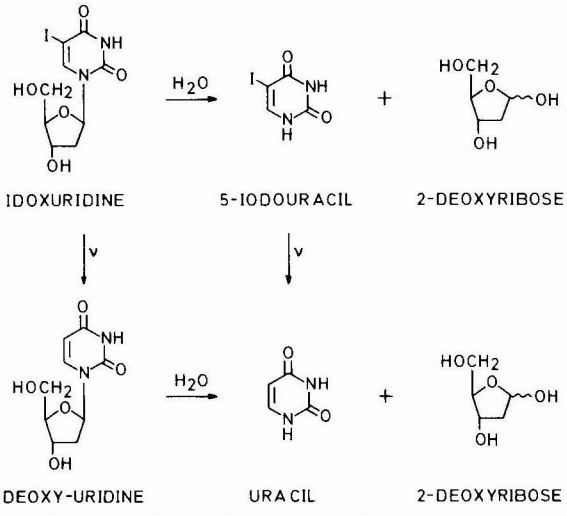

Fig. 1. The main degradation routes of idoxuridine.

to modify the official procedures in order to improve the selectivity and, if possible, the time of analysis. Several investigations over the last few years[2–7] have shown that reversed-phase packings of different origins have different selectivities and efficiencies, and that some packings are preferred for separation of certain classes of compounds. This behaviour is believed to be due primarily to differences in the relative proportion of hydrocarbon-bonded and free silanol groups, and to differences in the manufacturing processes leading to either monolayers or polymerized multilayers of the chemically bonded octadecylsilyl (ODS) phases.

For the present study ten different commercially available ODS silica packings and various eluent compositions were tested. By means of the calculated capacity factors, k', separation factors, α, and reduced plate heights, h, the possible gains in selectivity and elution time were evaluated. Investigations leading to the identification of one of the unknown peaks are also presented.

EXPERIMENTAL

Apparatus

The liquid chromatograph used consisted of an Altex Model 110 solvent metering pump, a Cecil 2012 spectrophotometer detector and a Rheodyne Model 7120 injection valve. Chromatograms were recorded on a Kipp & Zonen Model BD-8 recorder, and retention times and peak areas were measured by means of a Hewlett-Packard Model 3353 A laboratory data system.

Freeze drying of collected fractions was carried out after freezing in liquid nitrogen in a Heraeus Model RVT 220 vacuum oven at ambient temperature and at a pressure less than 10 Pa using a Trivac Model S2A vacuum pump. Mass spectrometry (MS) of the freeze-dried fractions was performed on a Finnigan Model 3100D/9500 instrument using direct sample insertion and the following instrumental settings: ion source temperature 250°C; electron energy 70 eV; and multiplier potential 2.3 kV.

Chemicals

5-Bromo-2′-deoxyuridine was obtained from Sigma (St. Louis, MO, U.S.A.). All other reagents were of analytical grade from E. Merck (Darmstadt, G.F.R.). The samples and preparations of idoxuridine were from different suppliers.

Chromatography

Stainless-steel columns (150 × 4.6 mm I.D.) were packed with the bulk ODS silica packings according to a previously described procedure[8]. ODS phases available only in pre-packed columns were used as obtained from the manufacturers. The efficiency of the columns, expressed as the reduced plate height, h, measured on naphthalene when eluted by 90% methanol in water, was determined for all the packings investigated. The columns were operated at room temperature, identically in every respect, apart from intentional modifications, to the procedure for related substances described in the BP 80 monograph on idoxuridine.

The separation on a semi-preparative scale was performed with a column (250 × 8 mm I.D.) packed with ODS-Hypersil and eluted with 5% acetonitrile in water at a flow-rate of 3 ml/min.

RESULTS AND DISCUSSION

The eluent

The BP 80 procedure for testing the content of related substances in idoxuridine using a μBondapak C_{18} column eluted with methanol–water (4:96 v/v) and sulphanilamide as internal standard was published by Carr[9]. The elution time is long, *ca.* 30 min, at the prescribed flow-rate of 1.7 ml/min. Furthermore, it is increased to 1–2 h if the procedure is carried out according to the BP 80 monograph on idoxuridine eye-drops which contain preservatives such as parabens.

In order to shorten the elution time, the amount and nature of the organic solvent in the eluent were changed. Table I shows the k' values for idoxuridine, its degradation products and sulphanilamide chromatographed on a μBondapak C_{18} column with different eluents. The order of elution of sulphanilamide and 2′-deoxyuridine is reversed when the methanol (MeOH) in the eluent is replaced by acetonitrile (MeCN) or tetrahydrofuran (THF). In the last systems the nucleosides are eluted faster, whereas the retention of sulphanilamide is affected only to a minor extent. The selectivity of the systems with 5% MeCN and 2% THF is equivalent to

TABLE I

k' VALUES FOR IDOXURIDINE, RELATED SUBSTANCES AND SULPHANILAMIDE OBTAINED ON A μBONDAPAK C_{18} COLUMN ELUTED WITH WATER CONTAINING DIFFERENT ORGANIC SOLVENTS

Substance	*Methanol*		*Acetonitrile*			*Tetrahydrofuran*			
	4%	*5%*	*3%*	*4%*	*5%*	*2%*	*3%*	*4%*	*5%*
(1) Sulphanilamide	1.3	1.2	1.0	0.9	0.8	1.1	0.8	0.7	0.6
(2) 2′-Deoxyuridine	2.1	1.8	1.0	0.7	0.5	0.6	0.4	0.2	0.1
(3) 5-Iodouracil	3.3	3.0	2.0	1.7	1.5	1.6	1.3	1.1	0.9
(4) Idoxuridine	11.7	10.1	6.3	4.9	3.5	3.5	2.4	1.8	1.4

TABLE II
k', α AND h VALUES FOR IDOXURIDINE, RELATED SUBSTANCES AND SULPHANILAMIDE OBTAINED ON VARIOUS ODS SILICA COLUMNS ELUTED WITH DIFFERENT ELUENTS
Substances numbered as in Table I. V_m is the hold up volume of the column.

Column	*Sub-stance*	*4% Methanol*			*5% Acetonitrile*			*2% Tetrahydrofuran*		
		k'	α	h	k'	α	h	k'	α	h
10-μm μBondapak C_{18}	1	0.9	1.8		0.7	1.7		0.7	1.7	
(300 × 4 mm),	2	1.6			0.4			0.4		
prepacked,	3	2.7			1.3			1.3		
V_m = 3.7 ml	4	9.7		20	3.2		28	2.9		28
10-μm Bio-Sil ODS	1	1.5	1.7		1.0	1.5		0.9	1.6	
(250 × 4 mm),	2	2.5			0.7			0.6		
prepacked,	3	4.1			1.8			1.7		
V_m = 2.6 ml	4	14.1		10	4.0		15	3.5		15
5-μm LiChrosorb	1	1.2	1.8		0.9	2.0		0.7	4.0	
RP-18	2	2.1			0.4			0.2		
(150 × 4.6 mm),	3	3.8			1.6			1.4		
V_m = 1.9 ml	4	14.2		20	3.5		33	2.5		111
5-μm Nucleosil C_{18}	1	2.0	1.5		1.3	1.6		1.2	1.6	
(150 × 4.6 mm),	2	2.9			0.8			0.8		
V_m = 2.2 ml	3	4.1			2.0			2.0		
	4	15.9		9	4.8		33	4.3		23
5-μm ODS-Hypersil	1	1.4	1.9		1.1	1.8		0.9	1.7	
(150 × 4.6 mm),	2	2.7			0.6			0.6		
V_m = 1.9 ml	3	4.4			1.9			1.7		
	4	17.3		6	4.3		10	3.4		12
10-μm Partisil ODS	1	1.0	1.5		0.7	1.1		0.8	1.3	
(250 × 4.6 mm),	2	1.5			0.6			0.6		
V_m = 3.4 ml	3	2.3			1.2			1.3		
	4	5.4		8	2.2		19	2.3		22
10-μm Partisil ODS-2	1	2.4	1.4		1.2	2.3		0.9	6.7	
(250 × 4.6 mm),	2	3.4			0.5			0.1		
V_m = 3.4 ml	3	6.5			2.2			1.6		
	4	22.4		62	4.6		130	3.0		115
10-μm Radial Pak C_{18}	1	2.4	1.1		1.5	2.9		1.2	3.0	
(100 × 8 mm),	2	2.6			0.5			0.4		
prepacked,	3	3.1								
V_m = 3.1 ml	4	13.1		8	2.6		130	2.8		16
5-μm Spherisorb S ODS	1	1.8	1.4		1.2	1.7		1.1	1.4	
(150 × 4.6 mm),	2	2.5			0.7			0.8		
V_m = 1.9 ml	3	3.9			1.9			1.8		
	4	12.4		11	4.0		22	3.6		18
6-μm Zorbax ODS	1	1.7	1.7		1.0	2.2		0.8	3.8	
(250 × 4.6 mm),	2	2.9			0.4			0.2		
prepacked,	3	5.3			1.7			1.5		
V_m = 3.0 ml	4	21.3		20	4.0		75	3.0		75

that of the system with 4% MeOH, but the total elution time is reduced to about one third.

The effect of the pH of the mobile phase was investigated by substituting 4% MeOH in water by 4% MeOH in 0.01 *M* potassium phosphate buffers with pH varying from 3.0 to 7.0. No influence of pH on the retention of any of the substances tested was seen in this range.

Octadecylsilyl silica packing

The suitability of a number of reversed-phase ODS silica packings for the separation in question was tested. In Table II are given the k' values for idoxuridine, its degradation products and sulphanilamide obtained with the prescribed eluent as well as with the above two solvents based on MeCN and THF. The differences in selectivity and efficiency are illustrated by the separation factor, α, for sulphanilamide and 2′-deoxyuridine and by the reduced plate height, h, measured with idoxuridine.

The results demonstrate the very different characteristics of ODS silica packings of different origin. Under the present conditions the compounds chromatographed show little retention on Partisil-10 ODS, which is only partly covered with ODS groups, compared to that on the other packings tested. The greatest retention is obtained on Partisil-10 ODS-2 and Zorbax ODS, both of which are declared to be of the monolayer type and with a high coverage of ODS groups. They give good resolution between sulphanilamide and 2′-deoxyuridine but the peaks are misshapen and display severe tailing. These two columns, however, have, as do all the others, a high efficiency equivalent to a reduced plate height, h, of 4–8, when operated in the reversed-phase manner as described under Experimental.

From Table II other chromatographic systems may be selected which enable a reduction of the elution time to about one third of the original. If the order of elution and thus the eluent is to be unchanged, Partisil-10 ODS is the preferred packing. Use of 5% MeCN in water as eluent on an ODS-Hypersil column affords an effective alternative.

Unknown impurities

During this study some samples of idoxuridine were found to contain unknown impurities. The one present in largest amounts was collected from a semi-preparative scale column and the fractions were freeze-dried. An electron impact mass spectrum of the impurity was recorded and is shown in Fig. 2 together with the mass spectrum of idoxuridine. The fragments with m/e = 306/308, 190/192 and 147/149 indicate a bromine compound. The fragment with m/e = 117 (deoxyribose) and other similarities to the fragmentation of idoxuridine strongly indicate the unknown impurity to be 5-bromo-2′-deoxyuridine. This was later confirmed also chromatographically by comparison with an authentic sample of the suspected compound.

Chromatograms of impure samples of idoxuridine using one of the above alternative systems are shown in Fig. 3. Other impurities than the one identified were present, especially in preparations, but in amounts too small for isolation and identification by this technique.

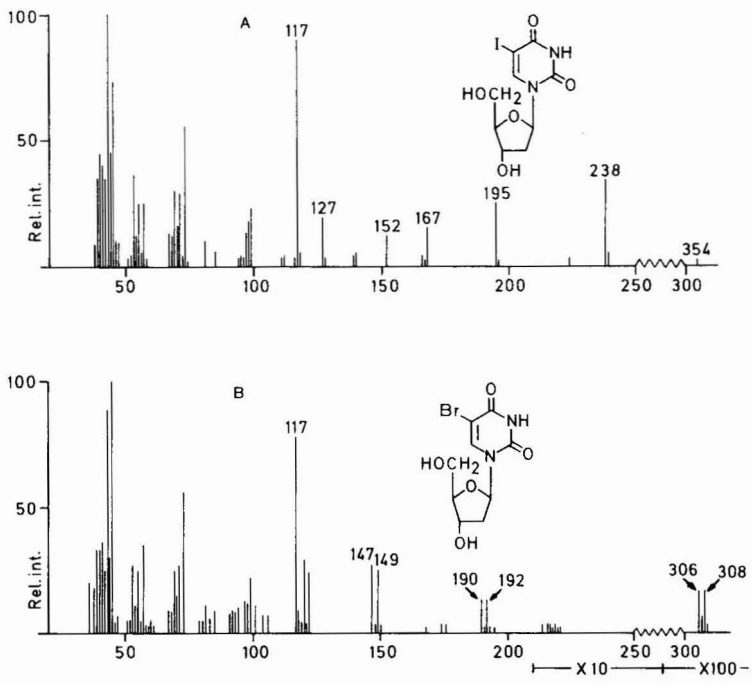

Fig. 2. Mass spectra (electron impact) of idoxuridine (A) and unknown impurity (found to be 5-bromo-2′-deoxyuridine) (B).

Sample preparation

The dissolution in water of even small amounts of idoxuridine takes a long time (12–24 h). The rate of dissolution cannot be increased by heating as the idoxuridine becomes partially hydrolyzed (Fig. 1). However, if the sample is dissolved in a

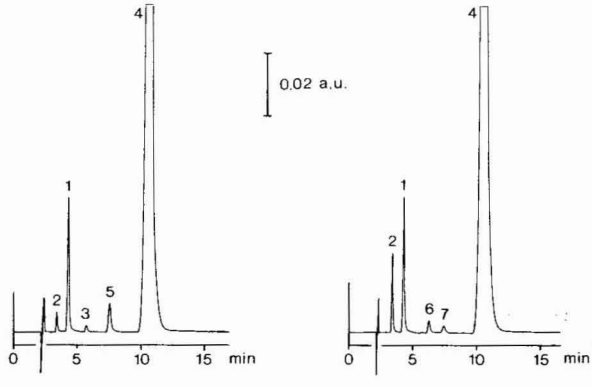

Fig. 3. Chromatograms of two commercial samples containing unknown impurities obtained on an 5-μm ODS-Hypersil column (150 × 4.6 mm) eluted with 5% acetonitrile in water, flow-rate 1 ml/min, with detection at 254 nm. Peaks: 1 = sulphanilamide; 2 = 2′-deoxyuridine; 3 = 5-iodouracil; 4 = idoxuridine; 5 = 5-bromo-2′-deoxyuridine (0.6%); 6 and 7 = unknowns.

small volume of dimethyl sulphoxide and the solution then diluted with water, sample preparation may be performed within a few minutes, thus minimizing the risk of biased results and reducing the overall time of analysis.

CONCLUSION

In official monographs, HPLC procedures may be specified in various degrees of detail. If the prescriptions involve a fixed composition of the eluent and a non-specific reference to a support (*e.g.*, ODS silica) difficulties may be expected due to the known differences in selectivity of packings of different origin. Consequently the specific brand of support which was used in the development of the procedure should be stated in the monograph. Better still, all brands which have been found suitable in a comparative study, such as the present, could be mentioned as validated possibilities.

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Note

Semiquantitative determination of trace substances in the ng/g (ppb) range by means of thin-layer chromatography

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By increasing the application volumes from microlitres to 5–50 ml per spot it is possible to detect and determine semiquantitatively trace substances in the ng/g (ppb) range by means of thin-layer chromatographic (TLC) techniques.

EXPERIMENTAL

Apparatus and method

A simple device allows the application of relatively large volumes onto silica gel 60 F_{254} Merck (Darmstadt, G.F.R.) thin-layer plates (Fig. 1). This method is based on known procedures, but adapted to the needs of TLC[1–8]. The continuous procedure is carried out under a permanent air draught in a fume-hood. The size of the spot is intentionally influenced by the rate of air circulation (adjustment of fume-hood window). The silica gel layer is marked and scratched with a metal pointer as shown in Fig. 2. Up to one blank and three standard samples may be spotted onto a single 200 × 200 mm plate.

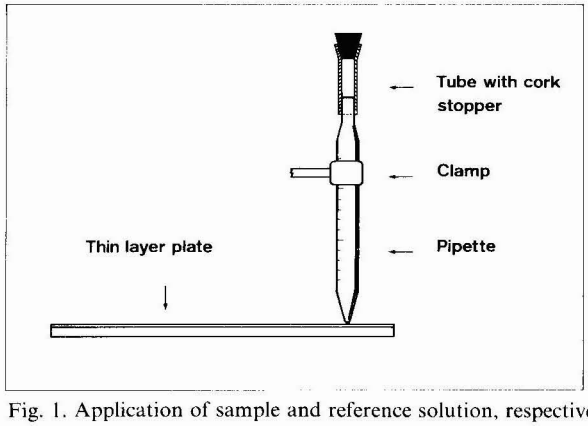

Fig. 1. Application of sample and reference solution, respectively.

Procedure

The marking lines on the plate are made by carefully and repeatedly scraping off the silica gel with a metal pointer (Fig. 2). The plate is then cleaned with a brush.

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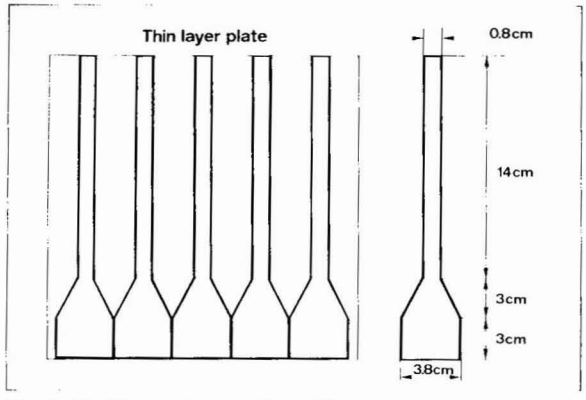

Fig. 2. Marking and separation of layer.

The sample is dissolved in a solvent with as low a boiling point as possible (*e.g.*, chloroform). The pipette is filled and closed at the top (Fig. 1). Excess of solution is removed by dispensing it onto a filter-paper until the pipette is filled to the mark.

The pipette is then placed at the start of the first track on the prepared thin-layer plate (Fig. 3). The pipette solution immediately runs out at a slow rate, so that the spotting of 20 ml of solution takes about 3–4 h in a self-controlled procedure. Constant surveillance is not necessary; however, occasional checking may be helpful. The spot must not be allowed to become too large otherwise substance is lost due to mass transport effects along the marking lines (Fig. 3). After spotting the plate, the spots are allowed to migrate once or twice with a polar mobile phase (*e.g.*, acetone) from the original into the second starting region (Fig. 3). Unwanted phenomena, such as formation of a ring of the concentrated substance and alteration of the R_F values due to impurities, are avoided by the following procedure.

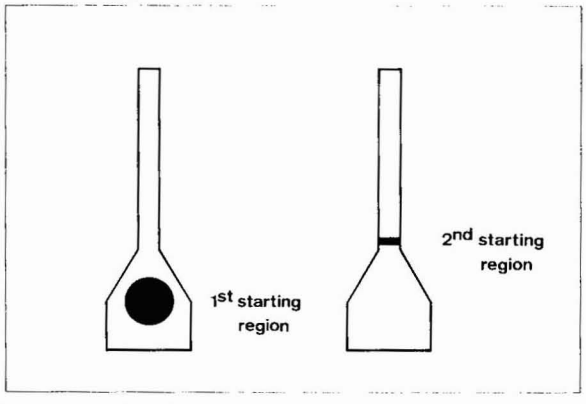

Fig. 3. Transfer of a single spot on a narrow silica gel strip.

After the substances are concentrated in the second starting region, both border lines near the spot are eluted with small quantities (*ca.* 10 μl) of the solvent used to concentrate the probes. With this method the tailing along the scratched lines

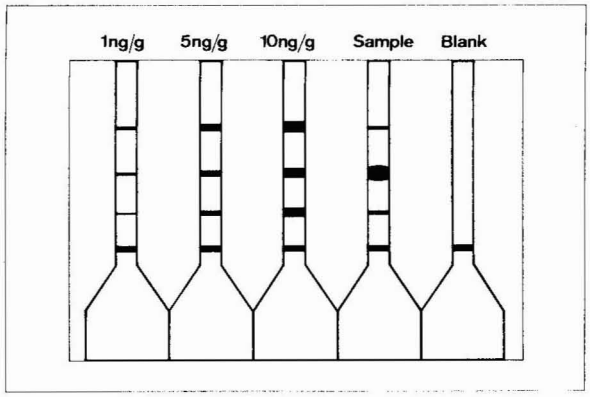

Fig. 4. Scheme of TLC separation with a mixture of dyestuffs. 1 = Irganol brilliant violet base; 2 = terasil brilliant red; 3 = cibazet violet; 4 = unknown.

is reduced or even completely avoided. After drying for a short time, the chromatogram is run in the usual way (Fig. 4). In order to ensure an optimum supply of mobile phase and to achieve a good separation, the plate must be immersed *ca.* 5 cm into the eluting solvent.

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Note

Feasibility study of high-temperature liquid crystals in wall-coated open-tubular columns*

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We previously reported[1–7] the synthesis and gas–liquid chromatographic (GLC) application of new high-temperature liquid crystals (mesophase transitions $>150°C$) for improved separations of isomers of chemical classes of practical importance. While we have demonstrated improved isomer separations of steroids[5], bile acids[6], and metabolic benzo[*a*]pyrene phenols[7], the principal thrust of our work had been focused on isomers of 3–5-ring polycyclic aromatic hydrocarbons (PAHs)[1–4].

The use of low-temperature liquid crystals (mesophase transitions $<150°C$) in wall-coated open-tubular columns had been reported earlier (*e.g.*, ref. 8) for low-molecular-weight aromatics (*e.g.*, disubstituted benzenes). The present study was undertaken to test the feasibility of high-temperature liquid crystals for PAH isomer separations when used as a wall coating substrate. N,N′-Bis[*p*-*n*-butoxybenzylidene]-α,α′-bi-*p*-toludine (BBBT) liquid crystal was selected for this study due to its ready solubility (in chloroform) for preparation of coating solutions, and its desirable temperature transitions[2].

EXPERIMENTAL

BBBT was prepared as reported previously[2], and was triple recrystallized from chloroform. The liquid crystal melted into a smectic phase at 159°, and had smectic–nematic and nematic–isotropic transitions of 188° and 303°, respectively. Wide-bore (0.76 mm I.D.) open-tubular columns were prepared from stainless-steel tubing (Handy and Harmon, Norristown, PA, U.S.A.), and were cleaned (chloroform, methanol, 50% potassium hydroxide solution, distilled water, 50% nitric acid, distilled water, methanol, chloroform), dried with nitrogen, and subsequently coated by a dynamic coating procedure at room temperature. The coated columns were con-

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ditioned in the gas chromatographic oven using a 1°/min temperature program to 220°C (with initial and final temperature hold times of 2–3 h each) and a helium carrier gas flow-rate of 5–10 ml/min. The PAH solutes were obtained from standard commercial sources and analyzed at four carrier gas (helium) flow-rates (3, 5, 7 and 10 ml/min) and six column temperatures. All solvents used were glass-distilled. GLC analyses were run on a Hewlett-Packard 7610A dual-flame gas chromatograph coupled to a Hewlett-Packard 3354 laboratory data system, with carrier gas flow-rate regulated with a calibrated mass flow controller. The GLC oven temperatures were calibrated by thermocouple readout to a calibrated temperature readout device. Sample volumes in the order of 0.3 μl or less were injected directly into an unmodified conventional inlet port, and attempts were not made to optimize the columns or chromatograph towards high chromatographic efficiencies.

RESULTS AND DISCUSSION

Retention data were obtained for 3-ring (fluorene, phenanthrene, anthracene) and selected 4-ring (triphenylene, benz[*a*]anthracene, and chrysene) PAH isomer mixtures on 165-m and 75-m wide-bore (0.76 mm I.D.) wall-coated BBBT columns in the nematic temperature range of the BBBT liquid crystal above 188°C. The PAH isomers in the 4-ring test mixture were selected since these three PAHs are often difficult to resolve on GLC high-efficiency wall-coated silicone columns[9] and HPLC C_{18}-bonded reversed-phase columns[10].

The resultant retention data, expressed as capacity factors (k'), are summarized in Table I. The retention data for 3-ring isomers are averages of the k' values determined at four carrier gas flow-rates (3.0, 5.0, 7.0, and 10.0 ml/min) for each isomer. The standard deviation of these average k' values ranged from 1–3%. Linear regression analysis of log k' *vs.* $1/T$(°K) for each isomer gave correlation coefficients exceeding 0.997. The separation factors (α) for consecutively eluting isomer pairs

TABLE I
CAPACITY FACTORS (k') FOR 3- AND 4-RING PAH ISOMERS ON BBBT LIQUID CRYSTAL OPEN TUBULAR COLUMNS

PAH Isomer	*k'*										
	Column temperature (°C)										
	195		*200*		*210*		*220*		*230*		*240*
	*Column**										
	A	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*A*
Fluorene	0.59	0.47	0.53	0.43	0.41	0.31	0.34	0.25	0.27		
Phenanthrene	1.53	1.21	1.34	1.07	1.02	0.77	0.79	0.62	0.63		
Anthracene	2.00	1.57	1.74	1.38	1.29	0.98	0.99	0.77	0.78		
Triphenylene				14.8		9.6	6.4	6.2		5.4	5.2
Benz[*a*]anthracene				20.6		13.0	9.7	8.2		7.0	6.6
Chrysene				26.4		16.5	12.9	10.4		8.7	8.0

* Column A: 165 m × 0.76 mm I.D.; Column B: 75 m × 0.76 mm I.D.

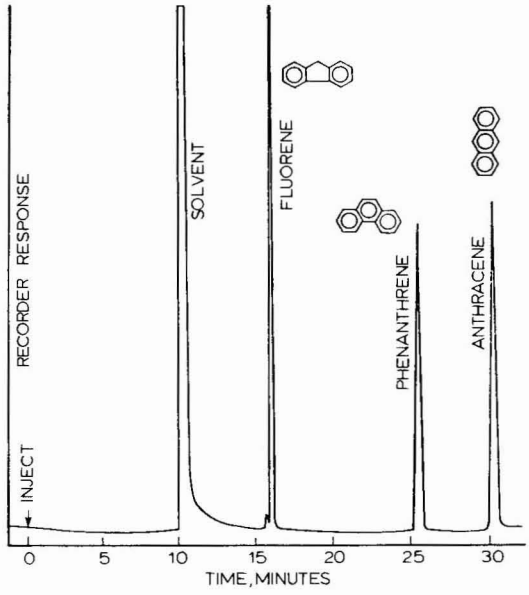

Fig. 1. Separation of 3-ring PAHs on BBBT wall-coated open-tubular column. Column: 165 m × 0.76 mm stainless steel, coated with BBBT; 195°C; carrier gas (helium) flow-rate 3 ml/min.

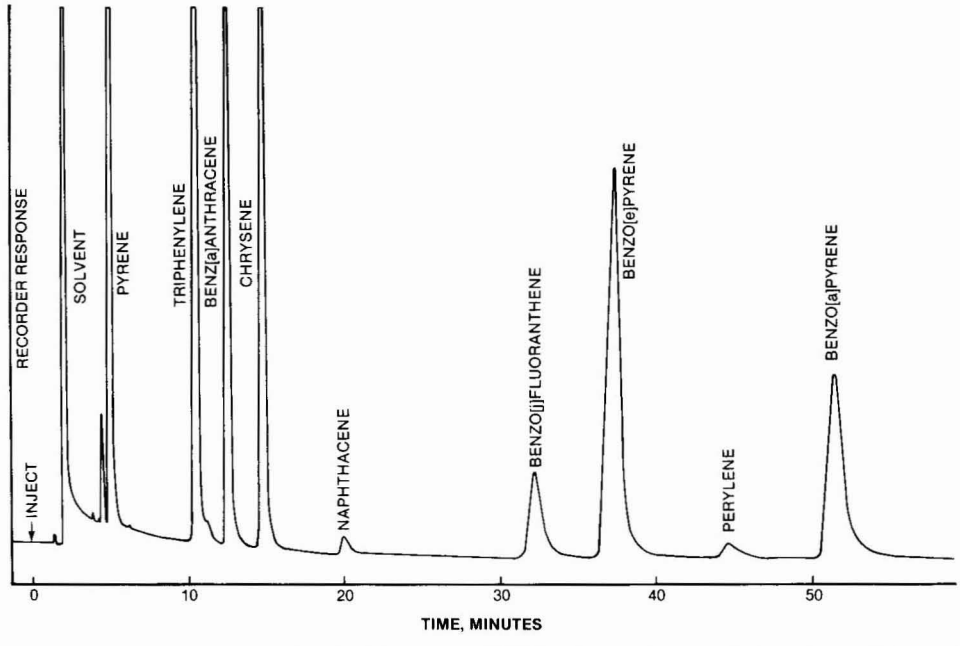

Fig. 2. Separation of 4- and 5-ring PAH isomers on BBBT wall-coated open-tubular column. Column: 75 m × 0.76 mm stainless steel, coated with BBBT; 240°C; carrier gas (helium) flow-rate 15 ml/min.

were in good agreement between the 165-m and 75-m columns, indicating little difference in relative selectivity as a function of column length. The temperature dependence of α values for isomer pairs was not large over the column temperature range studied, and useful α values were still attainable at the highest column temperatures used (*e.g.*, α[anthracene/phenanthrene] was 1.24 at 230°C; α[benz[*a*]anthracene/triphenylene] and α[chrysene/benz[*a*]anthracene] was 1.27 and 1.21, respectively, at 240°C). The separation characteristics of the wall-coated BBBT wide-bore open-tubular columns used in this study are illustrated in Fig. 1 for 3-ring PAHs (165-m column), and in Fig. 2 for a mixture of 4- and 5-ring PAH isomers (75-m column). These results are consistent with earlier findings for these isomers on packed columns[1–3], and offer evidence that a high-temperature liquid crystal used as a wall-coating substrate retains its selective capacity for the separation of PAH solute isomers.

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Note

Gas chromatographic separation of enantiomers of amines and amino alcohols on chiral stationary phases

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Capillary gas chromatography is a fast and reliable method for the assignment of the configurations of optically active compounds. Both the formation of diastereomeric derivatives and the direct separation of enantiomers on chiral stationary phases have been applied in many versions, especially for amino acids, amines, amino alcohols and α-hydroxy acids.

Most chiral stationary phases have poor thermal stability. This problem was overcome by Bayer and co-workers[1,2] by synthesizing polysiloxane stationary phases with chemically bound chiral constituents. A simpler approach was demonstrated by Saeed *et al.*[3,4], who succeeded in modifying commercially available silicone polymers with cyano groups in their side-chains such as OV-225 and Silar-10C. The cyano groups were converted by acid hydrolysis into carboxylic groups and acid chlorides and coupled with L-valine-*tert.*-butylamide. With these stationary phases they were able to separate amino acids and some amino alcohols such as norephedrine.

In this work we introduced chiral constituents into silicone OV-225 after reduction of the cyano groups to amino groups with lithium aluminium hydride ($LiAlH_4$) and by coupling benzyloxycarbonyl-L-valine and -L-leucine to the amino groups.

EXPERIMENTAL

Reduction of OV-225 with $LiAlH_4$

To a suspension of 50 mg of $LiAlH_4$ in 5 ml of dry diethyl ether a solution of 100 mg of OV-225 (Applied Science Labs., College Station, PA, U.S.A.) in 3 ml of diethyl ether was slowly added and the mixture was refluxed for 5 h. Excess of $LiAlH_4$ was then decomposed with water. After addition of concentrated sodium hydroxide solution the phases separated. The organic phase was removed, the aqueous phase was extracted three times with diethyl ether and the combined extracts were dried over sodium sulphate. After removal of the solvent, completion of the reaction was proved by infrared spectroscopy.

Coupling of Z-L-Val and Z-L-Leu to OV-225 amine

Z-Amino acids were prepared according to the procedure described by Grassmann and Wünsch[5] and added in equimolar ratio to the OV-225 amine in chloroform solution. After the addition of a small excess of dicyclohexyl carbodiimide (DCC)

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the reaction mixture was stirred at room temperature for 12 h. After completion of the reaction the solution was washed with 1 *N* hydrochloric acid, saturated sodium hydrogen carbonate solution and water and dried over sodium sulphate. To remove dicyclohexylurea the product was chromatographed on a silica column with ethyl acetate as eluent.

Preparation of glass capillary columns

Pyrex glass capillary columns were drawn on a Hupe and Busch drawing machine and coated as described previously[6]. Gas chromatography with hydrogen as the carrier gas was performed on a Carlo Erba Model 2101 gas chromatograph.

Formation of derivatives

Samples of 1 mg or less of amines or amino alcohols were dissolved in 200 μl of dichloromethane and trifluoroacetylated by addition of 50 μl of trifluoroacetic anhydride. After 30 min at room temperature the excess of reagent was removed with a gentle stream of dry nitrogen and the residue was dissolved in 100 μl of dichloromethane and used for gas chromatographic investigation. Amino acids were derivatized as reported earlier[7].

RESULTS AND DISCUSSION

Various intermolecular forces participate in the formation of diastereomeric complexes between a chiral solvent and chiral solutes in a gas chromatographic system. Amino acid derivatives in many instances have proved to be adequate constituents of chiral stationary phases for effective separations of enantiomers. It has been shown[8–10] that the structure of an acyl, ester or amide moiety of a stationary phase derived from amino acids or dipeptides strongly influences enantioselectivity. From our recent work[7,11] on the separation of α-hydroxy acids on mandelic acid derivatives as stationary phases we concluded that benzyloxycarbonyl residues may support enantiomer separation. It also seemed interesting to compare the properties of these new stationary phases with those prepared by Saeed *et al.*[3].

For these reasons we introduced chiral constituents into the silicone polymer OV-225 by reduction of the cyano groups to aminomethylene groups and by coupling Z-L-Val and Z-L-Leu to the newly formed amino groups with DCC as condensing agent (Fig. 1). As can be seen from the α-values in Table I, both the Z-L-Val and the Z-L-Leu phases give only poor separations of amino acid enantiomers. However, the separation factors for amino alcohols and amines are surprisingly high. Amino alcohols have repeatedly been identified as constituents of peptide antibiotics[12,13] and the determination of their configuration on a microgram scale has been difficult. The separation of trifuoroacetylated aliphatic amino alcohols and amines is not possible or very poor on such widely used chiral stationary phases as N-dodecanoyl-L-Val-*tert.*-butylamide[14], N-trifluoroacetyl-L-Val-L-Val-O-cyclohexyl ester[15] and Chirasil-Val[1]. Aliphatic, cyclic and aromatic amines were separated by Gil-Av and co-workers[16,17] on stationary phases derived from chiral aromatic amines and on carbonylbis(N-L-valine isopropyl ester)[18]. Good separation of the N,O-bis-trifluoroacetyl derivatives of DL-alaninol and DL-valinol was obtained on (O-benzyloxycarbonyl)-S-mandeloyl-L-valine-*tert.*-butylamide[7].

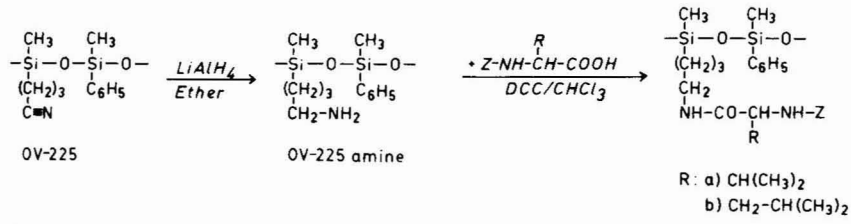

Fig. 1. Synthetic pathway for the preparation of chiral stationary phases.

TABLE I
α VALUES FOR CHIRAL STATIONARY PHASES

Racemate	*Z-L-Val-OV-225*		*Z-L-Leu-OV-225*	
	α value	*Column temperature (°C)*	*α value*	*Column temperature (°C)*
D,L-Alanine	1.00	70	1.013	80
D,L-Valine	1.026	80	1.015	80
D,L-*allo*-Isoleucine	1.011	80	1.00	80
D,L-Isoleucine	1.022	80	1.009	80
D,L-Leucine	1.017	80	1.00	80
R,S-2-Aminopentane	1.009	80	1.00	80
R,S-2-Amino-3-methylpentane	1.007	80	1.00	80
	1.010	80	1.005	80
R,S-2-Aminohexane	1.015	80	1.010	80
R,S-2-Amino-5-methylhexane	1.019	80	1.011	80
R,S-2-Aminoheptane	1.017	80	1.012	80
R,S-2-Amino-6-methylheptane	1.017	80	1.012	80
R,S-2-Aminooctane	1.020	80	1.015	80
R,S-Phenylethylamine	1.028	100	1.023	100
D,L-Alaninol	1.00	80	1.00	100
D,L-Aminobutanol	–	–	1.011	100
D,L-Valinol	1.008	80	1.018	100
D,L-Norvalinol	1.018	80	1.017	100
D,L-Leucinol	1.025	80	1.022	100
D,L-Norleucinol	1.023	80	1.019	100
D,L-Norephedrine	–	–	1.013	130
D,L-Mandelic acid	1.006	90	1.007	90

Chiral amino alcohols could also be separated as N-trifluoroacetyl-O-acyl derivatives[19,20] with propionyl, isobutyryl or pivaloyl as acyl residues. However, the procedure reported for the formation of derivatives does not seem to be easily applicable on a microgram scale.

As a peculiarity of the new chiral stationary phases the order of elution of amino acid enantiomers is not consistent. On the Z-L-Val phase the derivatives of D-Val, D-Ile and D-*allo*-Ile have longer retention times than the corresponding L-enantiomers.

With the Z-Leu-OV-225 stationary phase the derivatives of L-Ala but D-Val are retarded. Similar observations have been made with carbonylbis(N-L-valine isopropyl ester) as stationary phase[21]. With the amino alcohols (Fig. 2) the D-enan-

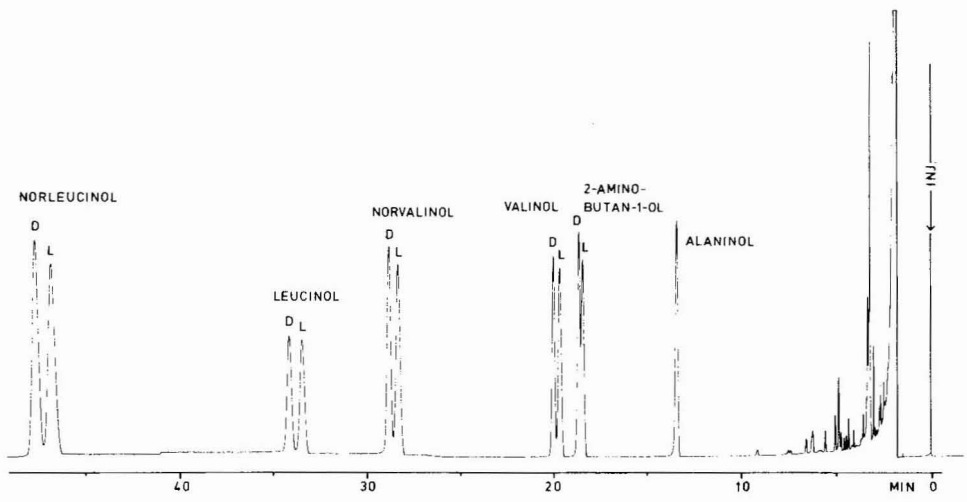

Fig. 2. Separation of N-trifluoroacetylamines on a Carlo Erba 2101 gas chromatograph with a 35 m × 0.2 mm I.D. Pyrex glass capillary coated with Z-L-Val-OV-225. Column temperature, 80°C; carrier gas, hydrogen (0.7 bar).

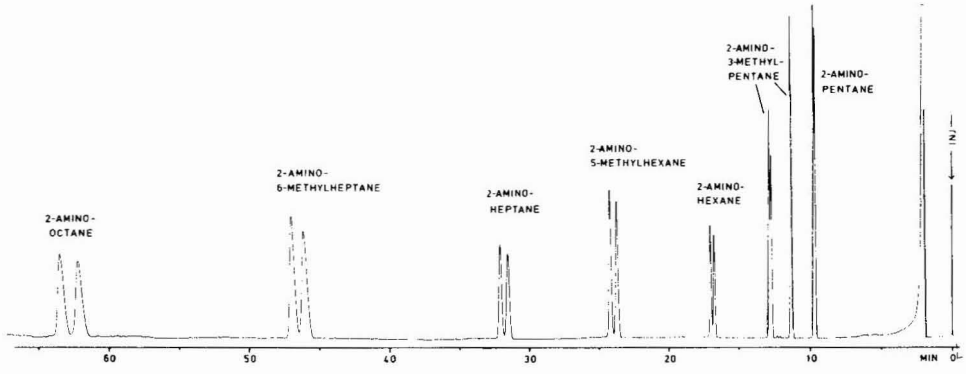

Fig. 3. Separation of N,O-bistrifluoroacetylamino alcohols on a Carlo Erba 2101 gas chromatograph with a 35 m × 0.2 mm I.D. Pyrex glass capillary coated with Z-L-Leu-OV-225. Column temperature, 100°C; carrier gas, hydrogen (0.7 bar).

tiomers consistently have longer retention times than the corresponding L-forms on both the Z-Val and Z-Leu phases. For the amines (Fig. 3) the S-enantiomers have longer retention times in the case of 2-aminopentane and phenylethylamine. The other 2-aminoalkanes were available only as racemates.

The major advantage of polymeric stationary phases over low-molecular-weight phases is their higher thermal stability. In this instance the limit of thermal stability has not yet been tested, but operation of the column at up to 130°C for several weeks did not decrease its separation efficiency. It is expected that further interesting results will be obtained by connecting other chiral compounds to functionalized silicone polymers.

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Note

Glass capillary gas chromatography of methyl, methyl 2-chloro and chloromethyl esters of C_2–C_{20} *n*-carboxylic acids

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Numerous papers has been published on the gas chromatography (GC) of carboxylic acids and their derivatives[1], but studies on their chloroalkyl esters have not been reported. On the other hand, the GC of fluorinated carboxylic acids and their methyl esters has been described[2,3].

Recently, the chlorination of short-[4], medium-[5] and long-chain[6] methyl esters have been reported to produce all possible monochloro isomers and also chloromethyl esters. GC product analyses indicated that the monochloro esters are eluted in sequence from 2-chloro to ω-chloroisomers, the 2-chloro esters always being eluted first. Chloromethyl esters left the column after 3-chloro[4] and 4-chloro isomers[5].

This paper describes a study of the GC of all methyl, methyl 2-chloro and chloromethyl esters of C_2–C_{20} *n*-carboxylic acids.

EXPERIMENTAL

Apparatus

GC analyses were carried out on a Varian Model 2400 gas chromatograph equipped with a flame-ionization detector and a 90 ft. × 0.012 in. I.D. glass capillary column coated with 5% Carbowax 20M, with a flow-rate of nitrogen of 1 ml/min. The column temperature was held at 40°C for 4 min, then programmed from 40 to 235°C at 8°C/min. The splitting ratio was 1:20. The temperatures of the injector and detector were 200 and 220°C, respectively.

The samples were purified by preparative GC on a 10 ft. × 3/8 in. O.D. aluminium tube packed with 10% Carbowax 20M on Chromosorb W (60–80 mesh). Appropriate temperatures were used, with a flow-rate of nitrogen of 100 ml/min.

Samples

Methyl esters were obtained by the usual sulphuric acid-catalysed esterification of commercial acids (Fluka, Buchs, Switzerland). Methyl 2-chloro esters were prepared from the corresponding 2-chloro acid chlorides[7] and methanol, except methyl chloroacetate, which was prepared from commercial chloroacetic acid (Fluka) by esterification. Chloromethyl esters[8] were synthesized from the corresponding acid chlorides and paraformaldehyde in the presence of a trace amount of zinc chloride.

The samples were purified, if necessary, by preparative GC and their structures were verified by NMR spectroscopy before GC analysis.

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RESULTS AND DISCUSSION

The gas chromatograms of methyl, methyl 2-chloro and chloromethyl esters are presented in Fig. 1, and the separation of a mixture of these esters is shown in Fig. 2. The retention data of the esters are given in Table I.

Polar stationary phases are more efficient than non-polar ones for the separation of isomeric monochloro esters. Even all eleven methyl monochlorododecanoates were resolvable in a Carbowax 20M glass capillary column[6].

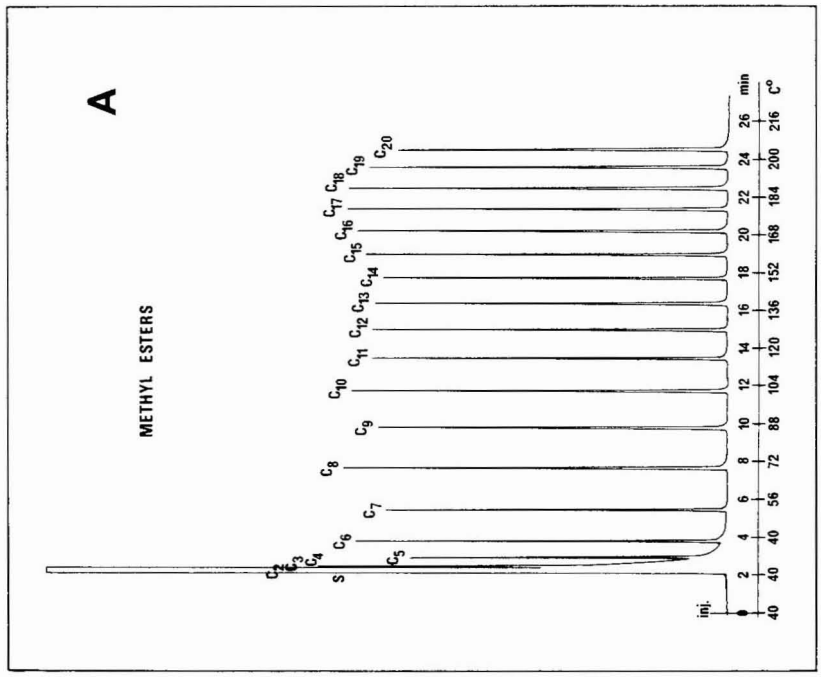
It is necessary to use temperature programming for the analysis of a mixture with a wide range of chain lengths. The initial temperature should be low enough to allow the short-chain derivatives to be separated from the solvent peak, and a sufficiently high subsequent temperature is required so that the long-chain isomers will be eluted sharply. This can be achieved by using a relatively short column, a rapid temperature programming rate and dilute samples.

All compounds were resolved, except methyl acetate, methyl propionate and partly also methyl butyrate, the peaks of which overlapped with that of the solvent. The separation of the mixture of esters was nearly complete; only methyl octanoate and chloromethyl pentanoate partly overlapped (Fig. 2).

TABLE I
RELATIVE RETENTION TIMES (RRT) FOR METHYL, METHYL 2-CHLORO AND CHLOROMETHYL ESTERS OF C_2–C_{20} *n*-CARBOXYLIC ACIDS

n-Carboxylic acid	*Retention time (min)**			*RRT***			*RRT****		
	Methyl ester	*Methyl 2-chloro ester*	*Chloro-methyl ester*	*Methyl ester*	*Methyl 2-chloro ester*	*Chloro-methyl ester*	*Methyl ester*	*Methyl 2-chloro ester*	*Chloro-methyl ester*
C_2	1.95[§]	5.92	3.93	1.00	3.04	2.02	0.16	0.37	0.24
C_3	2.10[§]	4.68	5.35	1.00	2.23	2.55	0.17	0.29	0.32
C_4	2.39	6.03	6.87	1.00	2.52	2.87	0.19	0.38	0.41
C_5	3.09	7.57	8.85	1.00	2.45	2.86	0.25	0.47	0.53
C_6	4.53	9.40	10.57	1.00	2.08	2.33	0.36	0.59	0.63
C_7	6.61	11.23	12.27	1.00	1.70	1.86	0.53	0.70	0.73
C_8	8.74	12.94	13.83	1.00	1.48	1.58	0.70	0.81	0.83
C_9	10.70	14.51	15.33	1.00	1.36	1.43	0.86	0.91	0.92
C_{10}	12.43	15.95	16.70	1.00	1.28	1.34	1.00	1.00	1.00
C_{11}	14.04	17.32	18.00	1.00	1.23	1.28	1.13	1.09	1.08
C_{12}	15.54	18.63	19.27	1.00	1.20	1.24	1.25	1.17	1.15
C_{13}	16.91	19.86	20.48	1.00	1.17	1.21	1.36	1.25	1.23
C_{14}	18.23	21.10	21.68	1.00	1.16	1.19	1.47	1.32	1.30
C_{15}	19.48	22.26	22.79	1.00	1.14	1.17	1.57	1.40	1.36
C_{16}	20.73	23.31	23.85	1.00	1.12	1.15	1.67	1.46	1.43
C_{17}	21.88	24.40	24.89	1.00	1.12	1.14	1.76	1.53	1.49
C_{18}	22.98	25.45	25.91	1.00	1.11	1.13	1.85	1.60	1.55
C_{19}	24.05	26.44	26.82	1.00	1.10	1.12	1.93	1.66	1.61
C_{20}	25.12	27.41	27.96	1.00	1.09	1.11	2.02	1.72	1.67

* From Fig. 2.
** Relative retention time for methyl esters taken as 1.00.
*** Relative retention time for C_{10} derivatives taken as 1.00.
[§] Retention times determined using methyl hexanoate as solvent.


A
METHYL ESTERS
S
C2
C3
C4
C5
C6
C7
C8
C9
C10
C11
C12
C13
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C15
C16
C17
C18
C19
C20
inj.
min
C°

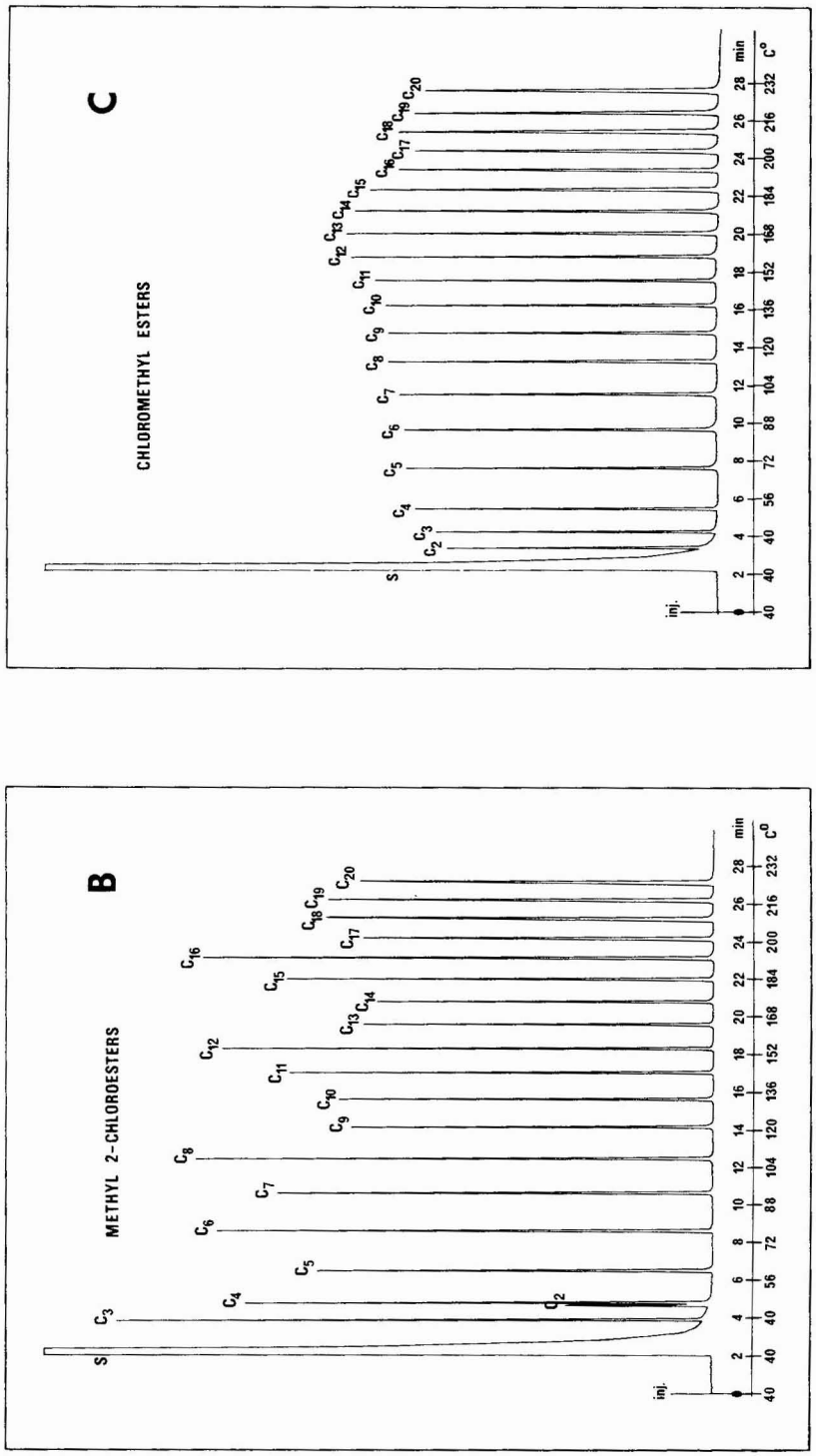

Fig. 1. Chromatograms of methyl (A), methyl 2-chloro (B) and chloromethyl esters (C) of C_2–C_{20} *n*-carboxylic acids.

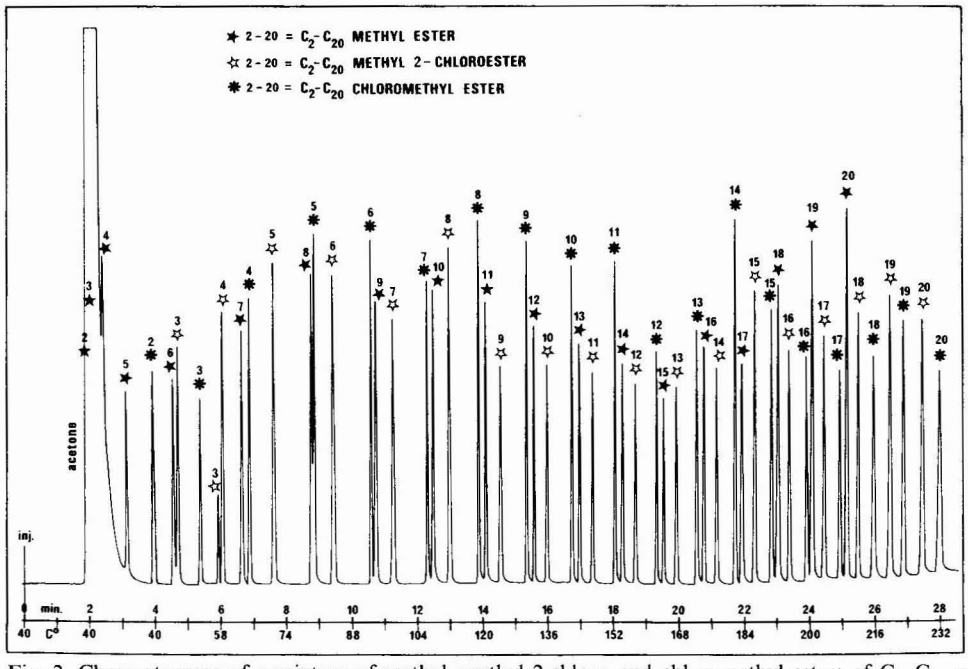

Fig. 2. Chromatogram of a mixture of methyl, methyl 2-chloro and chloromethyl esters of C_2–C_{20} *n*-carboxylic acids.

In general the samples left the column in the order methyl ester, methyl 2-chloro ester and chloromethyl ester, and in order of increasing chain length. With acetic acid derivatives, however, chloromethyl acetate, owing to its lower boiling point, was eluted before methyl chloroacetate. Also, methyl 2-chloropropionate was eluted before the latter compound.

Previous work[4–6] has indicated that all ω-chloro compounds have longer retention times than the other isomers. The terminal ω-chloro substituent seems to make methyl chloroacetate more polar than methyl 2-chloropropionate, which leads to opposite orders of elution on a polar column compared with methyl and chloromethyl esters. The retention times of methyl chloroacetate relative to methyl 2-chloropropionate on a polar Carbowax 20M and a non-polar OV-101 column were 1.26 and 0.78, respectively. The same effect has been reported with fluorinated acids and their methyl esters[2,3].

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Note

Identification of dieldrin derivatives

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Various derivatization reagents have been used for the identification of dieldrin, a chlorinated cyclodiene insecticide[1–5]. However, all of these methods were carried out in test-tubes and the different derivatives were identified by gas chromatography. Recently the derivatization of this insecticide on thin-layer plates and subsequent identification of the derivatives together with the parent compound were reported[6], and it was observed that dieldrin gave two spots due to derivatives; one of them was postulated to be dieldrin ketone but the other could not be identified. The present work shows that both spots are dieldrin ketones.

EXPERIMENTAL

All reagents were of AnalaR grade. The gas chromatographic apparatus and operating conditions were identical with those described previously[7]. Infrared spectra were recorded on a Beckman Acculab-1 instrument. Melting points were determined using micro heating table (Boetius) and were not corrected.

The derivatization of dieldrin was carried out on thin-layer chromatographic (TLC) plates by using zinc chloride–hydrochloric acid reagent as reported earlier[6]. The spots of derivatives at R_F 0.39 and 0.61 were scraped off and collected separately. The scrapings were extracted with *n*-hexane and the extracts were subjected to different tests.

An aliquot of each extract was evaporated to dryness and the melting points of the residues were found to be 155°C (R_F 0.39) and 105°C (R_F 0.61). Each extract was also analysed by gas chromatography and retention times were measured relative to lindane as the internal standard.

RESULTS AND DISCUSSION

Dieldrin on derivatization is known to be converted mainly to dieldrin ketone[2], and hence the predominant spot at R_F 0.39 (Table I) was previously postulated as dieldrin ketone. The data in Table I show that the extract of this spot and also that at R_F 0.61 gave positive responses with reagents such as 3,5-dinitrobenzoic acid and Schiff's reagent that are normally used for locating ketones, and the infrared spectra (KBr) of both extracts exhibited bands to OH (3450 cm^{-1}), C=O (1740 cm^{-1}) and ClC=CCl (1600 cm^{-1}. However, they had different relative retention times (6.8 and 5.9, respectively) and melting points (155 and 105°C, respectively).

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TABLE I
SUMMARY OF THE DIFFERENT TESTS CARRIED OUT ON EXTRACTS OF DIELDRIN DERIVATIVES

R_F value of extract of spot	*Test for ketones*	*Melting point (°C)*	*RRT**	*IR bands (cm^{-1})*
0.39	Positive	155	6.8	C=O (1740) OH (3450) ClC=CCl (1600)
0.61	Positive	105	5.6	C=O (1740) OH (3450) ClC=CCl (1600)

* Retention time relative to lindane.

Wienke and Burke[4] reported that treatment of dieldrin with zinc chloride–hydrochloric acid reagent normally gives a chlorohydrin via epoxide ring opening. Lombardo *et al.*[8] prepared aldrin chlorohydrin (melting point 150–152°C) by zinc chloride–hydrochloric acid method (yield 84%), with IR bands due to OH (3240 cm^{-1}) and ClC=CCl (1595 cm^{-1}). Dieldrin reacts with strong mineral acids to form a number of skeletally rearranged products. Originally Baker and Skerrett[9] used boron trifluoride etherate to produce "dieldrin ketone", which was also formed on using concentrated sulphuric acid[10]. However, all of these reactions yield a multi-component mixture (TLC giving at least three major spots with sulphuric acid and four with boron trifluoride–diethyl ether), although one product from the sulphuric acid reaction was isolated and exhibited IR bands due to C=O (1715 cm^{-1}), ClC=CCl (1602 cm^{-1}) and CH_2 (1368 cm^{-1}). The products from these reactions were collectively designated "dieldrin ketones"[10].

As aldrin chlorohydrin shows IR bands due to OH (3240 cm^{-1}) and ClC=CCl (1595 cm^{-1}) and both of the reaction products obtained in the present study show the presence of a C=O group, it appears that aldrin chlorohydrin formed *in situ* undergoes dehydrochlorination[10], resulting in the formation of two keto compounds. Moreover, the two keto compounds undergo reduction with zinc–hydrochloric acid, resulting in one compound (dieldrin). This indicates that dieldrin on derivatization with zinc chloride–hydrochloric acid on thin-layer plates was converted into two dieldrin ketones.

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Note

Preparative-scale high-performance liquid chromatography of ferricrocin, a microbial product*

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The advantages of high-performance liquid chromatography (HPLC) have been demonstrated in a large number of publications. Of interest is not only its analytical application but also the transformation of the analytical results to a preparative scale for isolation of substances of high purity. Such substances are needed as standards in analytical HPLC or for the elucidation of structure.

The preparative HPLC reported here was not carried out on an analytical column having a small diameter followed by collection of the fractionated eluate, but in large-diameter columns, as has been reported by other workers[1–4].

EXPERIMENTAL

Apparatus

A simple isocratic preparative HPLC system (Fig. 1) was constructed from a Haskel Model 26740 air-driven pump (Haskel, Burbank, CA, U.S.A.), a Rheodyne Model 7120 sample injector (Rheodyne, Berkeley, CA, U.S.A.) containing 100-μl and 2-ml sample loops and a Knauer Model 8100 spectrophotometric detector (Knauer, Berlin, G.F.R.), equipped with a preparative cell (pathway 2 mm).

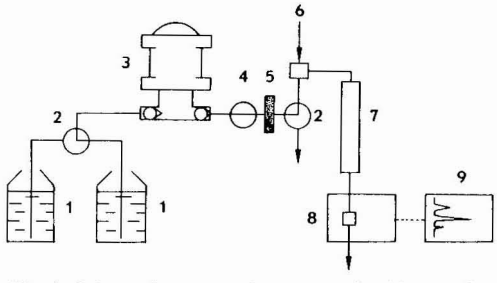

Fig. 1. Schematic set-up of a preparative high-performance liquid chromatograph. 1 = Solvent reservoir; 2 = three-way valve; 3 = pump; 4 = shut-off valve; 5 = solvent filter; 6 = injection valve; 7 = column; 8 = detector; 9 = recorder.

* No. 194 in the series Metabolic products of microorganisms.

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The reversed-phase columns packed with Merck LiChrosorb RP-8 (7 μm) (250 × 16 or 4.6 mm I.D.) were obtained from Knauer.

The Whitey valves, Nupro solvent filter and Swagelok fittings were purchased from Kontron (München, G.F.R.).

Reagents

Double distilled water was used. Acetonitrile GR was obtained from E. Merck (Darmstadt, G.F.R.).

Chromatographic conditions

Stationary phase: LiChrosorb RP-8 (7 μm). Mobile phase: water–acetonitrile (9:1); flow-velocity 12 cm/min. Column temperature: ambient. Detector: wavelength 270 nm; sensitivity, 2.0 absorbance units full scale.

Ferricrocin isolation

Ferricrocin, a metabolic product from *Aspergillus viridi-nutans* (Fig. 2), was isolated from the fermentation broth by adsorbing the culture filtrate on Amberlite XAD-2, as described recently[5]. The product was lyophilized and known amounts were dissolved in water and injected onto the HPLC column.

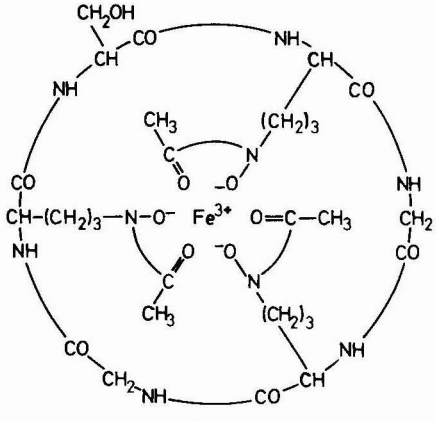

Fig. 2. Structure of ferricrocin.

RESULTS AND DISCUSSION

In an initial investigation, the efficiency of an analytical column was compared with the preparative column. When using the same particle size (7 μm), and the same sample weight and flow-rate relating to the column diameter, a reduction of about 10% was observed in the efficiency of the preparative column in comparison with the analytical column (Table I). This reduction is in contrast to results of Wolf[6] and Wehrli[7], who found a higher efficiency for the preparative column.

When the efficiency is determined by use of an ideal testing substance, such as naphthalene, the plate height will be reduced by a factor of 2. Ferricrocin is not an

TABLE I
EFFICIENCY OF THE ANALYTICAL AND PREPARATIVE COLUMNS

Column diameter (mm)	*Sample weight (mg)*	*Capacity factor, k′*	*Plate height, H (mm)*
4.6	1.0	3.8	0.078
16.0	12.5	4.0	0.087

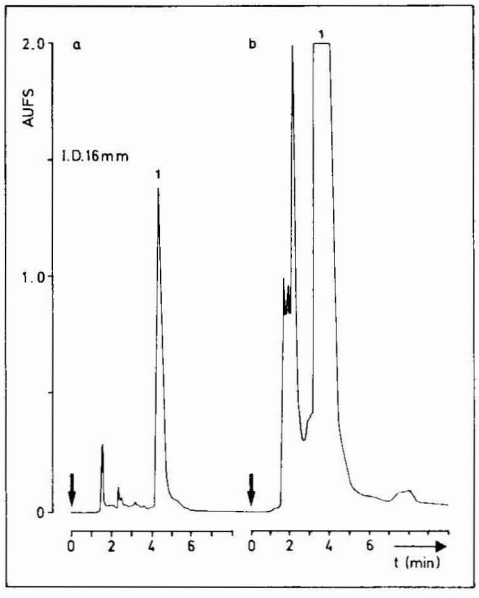

Fig. 3. Effect of column overload: a, 100 mg ferricrocin; b, 1 g ferricrocin. 1 = Ferricrocin.

ideal substance for determination of column efficiency because of its low molar extinction coefficient ($\varepsilon_{270\,nm} = 840\ l \cdot mol^{-1} \cdot cm^{-1}$) and polar character.

In a second investigation the loading limit of the preparative column was determined. Column overload in preparative chromatography is defined as a reduction in the k' values by 10% or more from the values for analytical separations[3]. This effect is shown in Fig. 3, when loading the column with 100 mg and 1 g, respectively. Table II demonstrates the dramatic reduction in the efficiency and increase in the plate height when column overload occurs. The k' value was reduced by 20% and the

TABLE II
COLUMN OVERLOAD

Sample weight (g)	*Capacity factor, k′*	*Plate height, H (mm)*
0.1	3.2	0.21
1.0	2.6	1.84

efficiency to 40% when a eight-fold sample weight (100 mg) was injected. When an 80-fold sample weight (1 g) was injected, the k' value was reduced by 35% and the efficiency to 5%.

In spite of a drastic reduction in the efficiency on column overloading, preparative HPLC is an ideal method for isolation of substances of high purity. Its advantage is founded on the short separation time which is derived from a high sample throughput and a high sample purity. In addition, the small peak volume is advantageous for the isolation of the separated substance from the eluent[2].

The capacity limit in the separation problem described above was reached with a sample weight of 20 mg per g stationary phase.

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Note

Reversed-phase high-performance liquid chromatography of substituted indoleacetic acids

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Methods using high-performance liquid chromatography (HPLC) for purification[1,2] and quantification[3] of indoleacetic acid (IAA) in plant extracts are now well established. In clinical chemistry HPLC is used for the determination of 5-hydroxyindoleacetic acid (5-OH-IAA)[4]. Recently, there has been considerable interest in substituted indoleacetic acids. 4-Chloroindoleacetic acid (4-Cl-IAA) and its methyl ester have been identified in immature seeds[5,6]. There are indications, although no conclusive evidence, that 5-OH-IAA is also present in plant tissues[7].

Both 5-OH-IAA and 4-Cl-IAA each show *ca.* 40% of the fluorescence intensity that IAA itself would show in the indolo-α-pyrone fluorescence determination after conversion into the respective α-pyrones[8]. This widely used procedure has proved to be a rapid, accurate[9] and reliable[10] method for quantification of IAA in plant extracts. However, 5-OH-IAA and 4-Cl-IAA must be excluded if the measured fluorescence is to be assigned only to IAA; this is not accomplished with the usual clean-up procedure[9]. Separation of IAA and 4-Cl-IAA seems to be difficult even by thin-layer chromatography (TLC) or gas–liquid chromatography (GLC)[8]. Thus, it was desirable to develop a quick, complete and easy separation of IAA, 5-OH-IAA, 4-Cl-IAA and its methyl ester in order to keep the fluorometric analysis of IAA reliable and to ease further research on substituted indoleacetic acids.

EXPERIMENTAL

Chemicals

IAA and 5-OH-IAA were purchased from Serva (Heidelberg, G.F.R.) and Sigma (St. Louis, MD, U.S.A.) respectively. 4-Cl-IAA and its methyl ester were a generous gift from K. C. Engvild, Risø National Laboratory, Department of Agricultural Research, Roskilde, Denmark. All chromatographic solvents were of high purity and redistilled before use.

Chromatographic equipment

Prepacked columns of 10-μm Spherisorb ODS (Phase Separations, Queensferry, Great Britain) and 7-μm LiChrosorb RP-8 (E. Merck, Darmstadt, G.F.R.) were used, of a size suitable for either preparative (250 × 10 mm I.D.) or analytical purposes (250 × 4.6 mm I.D.). Chromatography was carried out using an HPLC

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system (Laboratory Data Control, Riviera Beach, FL, U.S.A.) consisting of two pumps (Constametric II G), a gradient programmer (Gradient Master) and a fixed wavelength UV monitor (Model 1203, UV III) operating at 254 nm. Sample introduction was via a Rheodyne 7010 sample injector, injections of 800 μl being made into a 1-ml loop or of 50 μl into a 100-μl loop.

RESULTS AND DISCUSSION

The solvent systems used were binary mixtures of acidic buffers in water (0.1 *N* acetic acid) and methanol (0.1 *N* acetic acid in methanol). The addition of an acidic buffer masks residual adsorption sites on the stationary phase[2]. Spherisorb ODS is a spherical, totally porous silica with a C_{18} bonded stationary phase and LiChrosorb RP-8 is a similar but irregularly shaped material with a C_8 bonded phase. Both reversed-phase materials were suitable for separation of the four compounds (Figs. 1–3). Even with a convex gradient of increasing concentration of methanol, the compounds were well separated (Fig. 1). This gradient was applied to ensure an acceptable elution time for the least polar compound, the methyl ester of 4-Cl-IAA. Use of the preparative RP column as in Fig. 1 provides a rapid clean-up procedure. With crude plant extracts, addition of a guard column is strongly recommended.

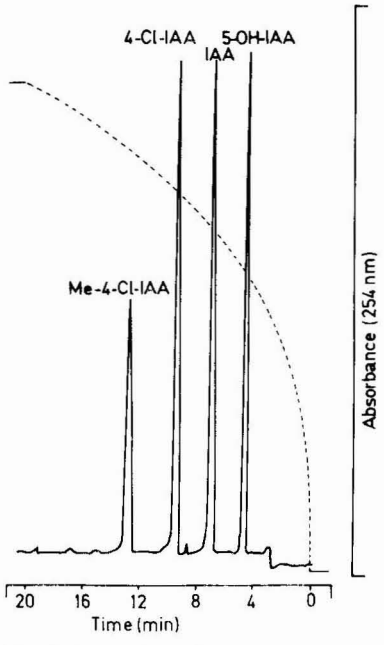

Fig. 1. Separation of IAA and substituted indoleacetic acids by reversed-phase HPLC. Column: 10-μm Spherisorb ODS (250 × 10 mm I.D.). Flow-rate: 5 ml/min. Mobile phase, convex gradient, 0.1 *N* acetic acid in water to 70% 0.1 *N* acetic acid in methanol over 20 min. Sample injected: 5-OH-IAA and IAA, 10 μg each; 4-Cl-IAA and Me-4-Cl-IAA, 20 μg each. Abbreviation: Me-4-Cl-IAA = methyl ester of 4-Cl-IAA.

The elution sequence was the same for LiChrosorb RP-8 columns (Figs. 2 and 3). With analytical columns, excellent separation was obtained with a linear gradient

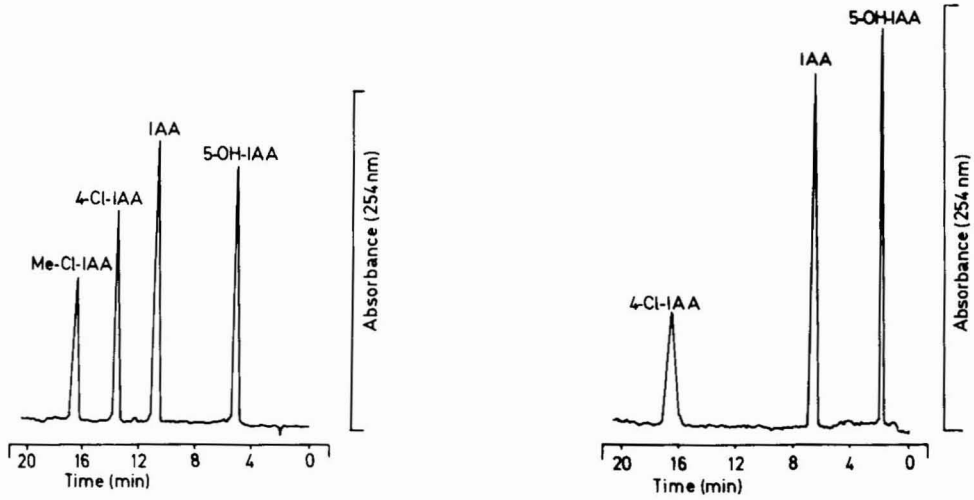

Fig. 2. Separation of IAA and substituted indoleacetic acids by reversed-phase HPLC. Column: 7-μm LiChrosorb RP-8 (250 × 4.6 mm I.D.). Flow-rate: 1.5 ml/min. Mobile phase, linear gradient, 20% 0.1 *N* acetic acid to 70% 0.1 *N* acetic acid in methanol over 15 min. Sample injected: 5-OH-IAA, 250 ng; others, 500 ng.

Fig. 3. Separation of IAA and substituted indoleacetic acids by reversed-phase HPLC. Separation conditions as in Fig. 2 except mobile phase: isocratic, 35% 0.1 *N* acetic acid in methanol.

(Fig. 2). As with the Spherisorb ODS column, rather steep gradients had to be used to achieve separations in a reasonable time. Shorter column lengths would also be suitable. Although 4-Cl-IAA is expected to be more polar than IAA it was retained longer than IAA. On the other hand 5-OH-IAA was retained only for a short time.

The analytical RP-8 column can be used in an isocratic mode if the main purpose is to recover IAA from its substituted compounds (Fig. 3). This is especially important if IAA is to be quantified by the indolo-α-pyrone method[8]. 5-OH-IAA and 4-Cl-IAA, which both interfere with that method, can easily be excluded, since 5-OH-IAA is eluted and the methyl ester of 4-Cl-IAA is retained on the column.

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Note

High-performance liquid chromatography of *cis*- and *trans*-en-in-di cyclo ethers (spiro ethers) in *Matricaria chamomilla* L. flowers and i chamomile extracts

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The *cis*- and *trans*-en-in-dicyclo ether 2-hexa-2,4-diin-1-ylidene-1,6-dioxa spiro[4,4]non-3-ene, (1 and 2; Fig. 1), have been isolated from some species c Matricaria[1] and some preliminary pharmacological investigations have been per formed[2–4]. Apparently, only the *cis* isomer exerts a spasmolytic action, which is a least ten times higher than that of papaverine[3,8]. The separation of (1) and (2) i chamomile has been accomplished by thin-layer chromatography[5], gas–liquid chro matography (GLC)[6] and liquid chromatography on Kieselgel columns[7].

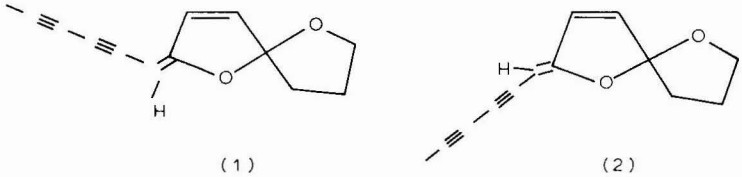

Fig. 1. Structures of the spiro ethers. 1 = *cis*-En-in-dicyclo ether; 2 = *trans*-en-in-dicyclo ether.

High-performance liquid chromatography (HPLC) seemed to be the metho of choice for the analysis of these compounds, owing to the simple preparation of th sample and the mild conditions of operation. After preliminary runs, we found tha on a reversed-phase column an excellent separation of (1) and (2) is possible, an detection at 317 nm ensures high sensitivity owing to the high absorbance maximun (ε = 19,500 l mol^{-1} cm^{-1}).

EXPERIMENTAL AND RESULTS

Dicyclo ethers are present almost exclusively in the receptacle of the inflores cence, where (−)-α-bisabolol and its oxides are not found. Pure (1) and (2) have beer prepared by extracting receptacles of *Matricaria chamomilla* L. flowers with di chloromethane and separating them on a silica gel column (*n*-hexane–ethyl acetate 97:3). GLC analysis showed that the (1) and (2) were 99.1 % and 98.1 % pure, respec tively.

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Routine analysis of the flowers is performed by stirring in methanol (10 ml) 1 g of pulverized flowers (2 h, room temperature) and injecting 5 μl of the solution after filtration. Analysis of water–alcohol extracts is performed by direct injection of 5 μl of the extract. A Perkin-Elmer Series 3 liquid chromatograph equipped with a microprocessor-controlled pump module, a Rheodyne injector, a Model 023 recorder, a Model 55B UV–visible detector and a 25 × 0.26 cm I.D. HC-ODS Sil-X reversed-phase column (Perkin Elmer) was used. A Minigrator integrator (Spectra-Physics, Santa Clara, CA, U.S.A.) was employed. The operating conditions for HPLC were as follows: room temperature; eluting solvent, acetonitrile–water containing 2% of acetic acid (2:3); flow-rate, 1 ml/min; wavelength of UV detector, 317 nm; recorder chart speed, 1 cm/min; sample size, 5 μl; peak width parameter value of integrator, 5; slope sensitivity, 250.

The quantitative determination of spiro ethers in unknown samples under the above conditions was studied using external standardization, and excellent precision and reliability were obtained. The spiro ethers were well separated, giving retention times ($t_R - t_0$) of 3.2 min (*trans*-en-in-dicyclo ether) and 5.2 min (*cis*-en-in-dicyclo ether) (Fig. 2).

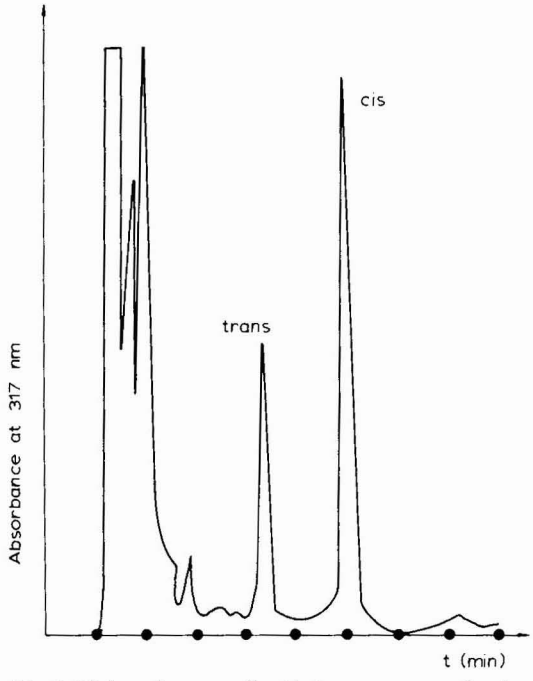

Fig. 2. High-performance liquid chromatogram of a chamomile extract.

DISCUSSION

Previous work has shown that HPLC is an excellent method for the determination of flavonoids[8,9] and coumarins[10] in *Matricaria chamomilla* L. flowers and chamomile extracts. The method has also been applied satisfactorily to dicyclo ethers

as a result of its simplicity, sensitivity and mildness of conditions. Analyses were carried out at room temperature, thus preventing possible *cis–trans* isomerization at elevated temperature, and the contents of (1) and (2) in chamomile extracts were determined. However, a systematic study of the stability of dicyclo ethers is required as the available data provide no information on this aspect. Finally, it should be possible to apply the method to the determination of dicyclo ethers and other components in chamomile infusions.

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Note

Separation of 2-amino-3*H*-phenoxazin-3-one impurity from *o*-aminophenol*

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o-Aminophenol (OAP) is an important intermediate for dyes, drugs and pesticides, and for the last two applications it must be highly pure. For use in the production of pesticides such as phosalone, the content of phenoxazone-type coloured impurities in OAP should be lower than 0.5%, otherwise the purity and yields in subsequent stages are affected. A spectrophotometric method for the determination of phenoxazone impurities in commercial OAP has been reported by us[1]. The presence of phenoxazone ring system in dyes and biologically active compounds such as actinomycins has created considerable interest in the synthesis and chemistry of phenoxazones[2–4]. The isolation of aminophenoxazone derivatives from microorganisms, insects, mammalian tissues and plants has been reported[5–8].

The identification of 2-amino-3*H*-phenoxazin-3-one (APZ) as the product of the oxidation of OAP with hydrogen peroxide in the presence of chromium(VI) has been reported[9]. However, so far no such phenoxazones have been isolated as impurities from commercial OAP obtained by catalytic hydrogenation of *o*-nitrophenol (ONP). We detected the presence of trace amounts of APZ by high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC). Different solvents such as chloroform–acetone, chloroform–ethyl acetate, chloroform–acetic acid and ethyl acetate–acetic acid have been suggested[10] for the TLC separation of APZ and its derivatives obtained by the oxidation of OAP and several other substituted phenols. However, R_F values for APZ (isolated from ONP reduction products) using different solvent systems have not been reported. We therefore decided to select a suitable solvent system to improve the separation and isolation of APZ from OAP.

EXPERIMENTAL AND RESULTS

Hydrogenation of ONP

The hydrogenation of ONP (50.0 g) was carried out in a stainless-steel autoclave (Parr type with stirring motor and internal cooling coil) of 2 l capacity using distilled methanol (1 l) as solvent. The pressure was kept at 140 p.s.i.g. and the temperature was maintained at 85°C for 2 h. Raney nickel (Type W-4; 0.5 g) was employed as the catalyst. The course of the reaction was followed by removing

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aliquots at different time intervals through a dip tube under a positive pressure and determining the unreacted ONP volumetrically using titanium(III) chloride[1].

At the end of the reaction the catalyst was allowed to settle and the supernatant solution was siphoned out under positive pressure in a round-bottomed flask containing a stabilizer (hydrazine hydrate, 1.0 ml), through which was passed a continuous stream of nitrogen. One gram of the solid obtained was dissolved in 20.0 ml of concentrated hydrochloric acid. Spectrophotometric assay[1] of the product at 485 nm indicated the presence of 4.0 mg of APZ per gram of the product.

HPLC procedure

HPLC was performed on a Waters Model ALC/GPC-202R401 liquid chromatograph. The system consisted of a UV detector (280 nm) and a dual pen recorder. A 30 cm × 4 mm I.D. μBondapak C_{18} column (Waters Assoc., Milford, MA, U.S.A.) was used. Methanol–water (1:1) was used as the solvent at a flow-rate of 1.5 ml/min and the chart speed was 0.75 in./min. Aliquots (10 μl) were chromatographed and the chromatogram showed two peaks with retention times of 2.9 and 3.6 min corresponding to OAP and APZ, respectively.

TLC analysis

The following solvent systems were tried, using standard conditions on 18 × 4 cm plates coated with 0.5 mm thick layers of silica gel G: (A) benzene–ethyl acetate (9:1); (B) chloroform–ethyl acetate (9:1); (C) chloroform–acetic acid (9:1); (D) chloroform–acetone (9:1); and (E) ethyl acetate–acetic acid (9:1).

Solvents. All solvents were of analytical-reagent grade and were dried and redistilled. They were degassed by refluxing under stirring, followed by cooling in the presence of nitrogen.

Materials. An authentic sample of APZ was prepared by the oxidation of OAP with *p*-benzoquinone[11].

Sample solutions. OAP, APZ and the solid obtained from the reduction of ONP were accurately weighed and dissolved in methanol to the desired volume (concentrations: OAP, 50.0 mg/ml; APZ, 0.1 mg/ml).

Spotting the plates. The sample solutions were spotted on TLC plates using a disposable capillary. The solvent was allowed to migrate to a height of 10 cm above the point of application, then the plate was dried. The spots corresponding to APZ and OAP were easily visible owing to their colours (pale yellow and dark brown, respectively).

Preparative TLC

The R_F values for APZ and OAP using different solvent systems are given in Table I. System C (chloroform–acetic acid, 9:1) afforded the best separation, giving a separation distance of at least 41 mm between the inner edges of the bands of APZ and OAP. Chromatograms run with systems A, B and D showed a tendency to form elongated and diffuse spots, especially that of OAP.

o-Aminophenol (2.0 g) obtained from the hydrogenation mixture dissolved in 40.0 ml methanol was spotted on each of 40 glass plates (20 × 20 cm); the spotting on each plate was completed within 5 min and the plates were further dried for 5 min only. The development was carried out in a glass jar (25.5 × 12 × 25 cm) at room

TABLE I
R_F VALUES OF OAP AND APZ IN DIFFERENT SOLVENTS
R_F values (× 100) are reported as the means of triplicate determinations. Plates were developed in an iodine chamber.

Compound	*Solvent system*				
	A	*B*	*C*	*D*	*E*
OAP (pure)	25	12	9	16	32
OAP (from reduction mixture)	25	12	9	16	32
APZ	29	25	73	38	68

temperature. The development chamber was saturated with nitrogen and the solvents used for development were degassed. Ascending development to a height of 10 cm was completed in 12 min. The bands corresponding to APZ and OAP were easily visible owing to their colours and were scraped off the plate into individual small glass columns fitted with sintered-glass discs. The APZ and OAP were extracted from the adsorbent with ten 5-ml portions of degassed methanol. The solvent was removed by evaporation under nitrogen and the solids obtained were weighed. This method of separation yielded 7.8 mg of APZ (95% recovery) from OAP. The product APZ had HPLC retention time, IR spectrum and melting point identical with those of an authentic sample and its elemental analysis was in agreement with its structure [ν (Nujol) 3250, 3300, 1653, 1600, 1470, 1389, 1274, 1219 and 1176 cm^{-1}; m.p. 249°C, lit.[11] 249°].

Fig. 1 shows an example of the chromatographic distribution of APZ and OAP.

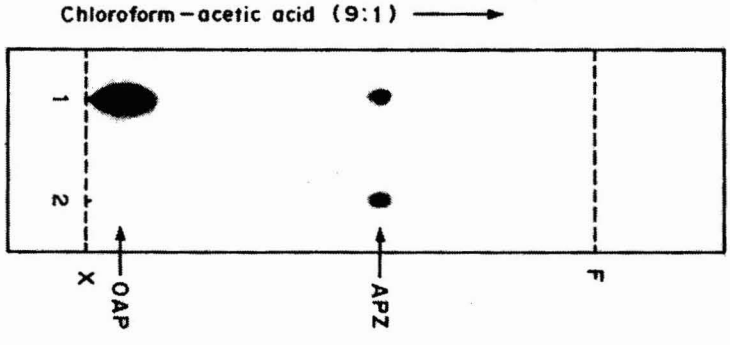

Fig. 1. Thin-layer chromatogram developed with chloroform–acetic acid (9:1). X = origin; F = solvent front. Lanes: 1 = reduction product (OAP); 2 = authentic sample of APZ.

Similar results were obtained when considerably larger volumes of sample (preparative TLC) were spotted. This rapid and one-step elution technique for the separation of APZ enables the amount of APZ formed in OAP to be measured quantitatively and serves as a quality control test for OAP obtained by the hydrogenation of ONP.

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Note

Determination of dothistromin by quantitative reversed-phase thin-layer chromatography

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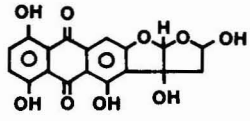
(Received January 13th, 1981)

Dothistromin (1) is the major metabolite produced by the pine needle pathogen *Dothistroma pini* (Hulb.) grown in culture[1–3], and the sole metabolite identified in extracts of diseased pine foliage. During studies on the effect of dothistromin on pine needle tissue, large numbers of samples were required to be analysed for the quantity of dothistromin injected into pine needles, for residual dothistromin after various physiological treatments of seedlings or detached pine needles, and also for dothistromin in natural disease lesions[4].



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Previously, dothistromin has been routinely analysed in this laboratory by adsorption thin-layer chromatography (TLC) and fluorescence densitometry (silica gel GF, developed in ethyl acetate–dichloromethane (1:1) containing *ca.* 4% formic acid to suppress dothistromin ionisation; dothistromin R_F 0.5). Ethyl acetate solutions of dothistromin exhibit a fluorescence excitation maximum at 470 nm and an emission maximum at 550 nm. When dothistromin is adsorbed on to a silica gel G TLC plate, the excitation maximum appears at 436 nm (Fig. 1). This method has been satisfactory for analysis of dothistromin extracted from fungal cultures, bioassays, and from dead, brown pine needles, but did not give reliable quantitative analyses of dothistromin extracted from green pine needles. Extraction of green pine needles containing very small amounts of dothistromin also released comparatively large amounts of chlorophylls and phenols. These on adsorption TLC migrated to R_F 0.7–0.9 but with variable streaking, which appeared as a faint blue fluorescence when examined at 366 nm, and quenched silica gel 254 nm fluorescence in addition to causing variable quenching of dothistromin fluorescence, making the analysis unusable for green tissue extracts.

This problem has been overcome by using a cheap, simple reversed-phase TLC system described in this paper.

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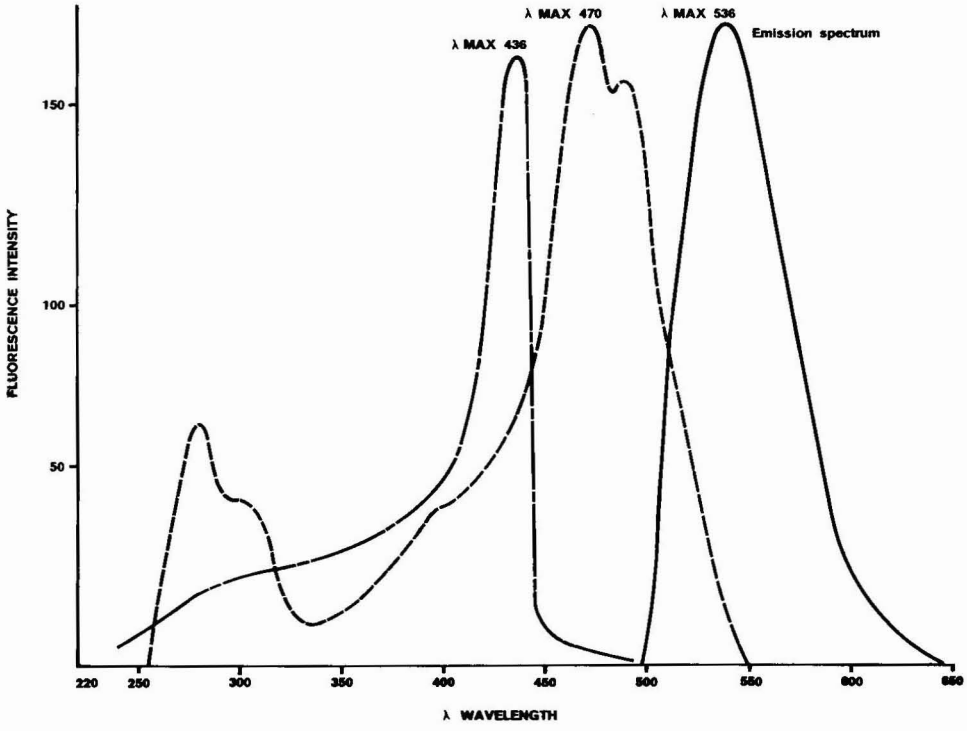

Fig. 1. Fluorescence excitation and emission spectra for dothistromin. ——, Adsorbed on to silica gel G; – – –, in ethyl acetate.

EXPERIMENTAL

Solvents were laboratory-reagent grade. TLC plates were prepared by spreading silica gel GF_{254} (Woelm, Eschwege, G.F.R.; 25 g) slurried in distilled water (55 ml) on five 20 × 20 cm glass plates. Layer thickness 0.25 mm. Plates were allowed to air-dry at 20°C for 12 h. Reversed-phase TLC plates were prepared by developing the silica-gel GF plates once in a solution of 5% liquid paraffin (BDH, Poole, Great Britain; infrared-spectroscopy grade) in hexane, and then air-drying[5]. The solvent system for reversed-phase TLC was methanol–water (2:1) containing *ca.* 4% formic acid. Vapour phase saturation was not used. Both solvent developments were in the same direction.

Dothistromin standards in ethyl acetate were prepared in the range 1–100 mg/l. Natural *Dothistroma* lesions and dothistromin-induced lesions in pine needles were excised (*ca.* 5 mm needle length; 1.8 mg fresh wt. tissue) and cut into *ca.* 0.5-mm pieces with fine scissors directly into 25-ml vials. Ethyl acetate (1 ml) and formic acid (50 μl) were added, the vials capped, and kept at 20°C for 12 h. The light green solutions were mechanically separated from tissue fragments and transferred quantitatively (Pasteur pipette) into 1-ml tapered vials and evaporated in a current of air at 20°C. The residue was dissolved in ethyl acetate (25 μl).

Samples (25 μl) and standards (10 μl) were applied to the reversed-phase TLC plates using a liquid-tight stepper-motor-driven 50-μl microsyringe.

After development the TLC plates were allowed to air-dry completely and analysed using a Vitatron TLD 100 densitometer with the following parameters: Hg light source, primary interference line filter λ 436 nm, secondary blocking filter $\lambda >$ 500 nm, slit 1 mm, oscillation 14 mm, scan speed 10 mm/min, photomultiplier sensitivity 100. The raw signal was minimally corrected for positive baseline drift (1.5% full scale/h) and baseline noise (2% full scale) (Kontes Corrector K-495100). Peaks were integrated (Hewlett-Packard 3371 B integrator).

RESULTS AND DISCUSSION

When extracts of dothistromin-treated pine needles were chromatographed using reversed-phase TLC as above, dothistromin migrated to R_F 0.6. The solvent front was allowed to travel 10 cm from the point of sample application. Chlorophylls remained at the origin, identifiable by their deep red fluorescence when irradiated at 436 nm and examined through a 500-nm blocking filter. Blue-fluorescent compounds appeared close to the chlorophylls at $R_F < 0.1$, and also close to the solvent front at $R_F > 0.9$. No fluorescence was detectable from R_F 0.2–0.9 in chromatograms of green, healthy, pine needle extract. Despite the samples and standards being applied to the reversed-phase TLC plate in ethyl acetate (which must initially also dissolve the paraffin coating on the silica gel), no adsorption of dothistromin on to the silica gel at the application site was observed, since after chromatography of 100 ng of dothistromin, no fluorescence was detectable at the origin. Dothistromin migrated on reversed-phase TLC as a compact spot, and a typical response from 50 ng is shown in Fig. 2.

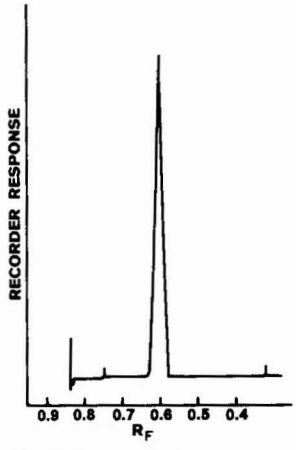

Fig. 2. Reversed-phase thin-layer chromatogram of 50 ng dothistromin.

The weight–response log–log plot for dothistromin standards is shown in Fig. 3, and is linear over the range 10–150 ng. This is distinct from the weight–response curve determined for adsorption TLC, where linearity extended over the range 10–700 ng. It is apparent that self-quenching of the dothistromin spot where quantities are greater than 200 ng is due to its slower lateral diffusion rate on reversed-phase TLC compared with adsorption TLC. The small linear range is a disadvantage of the

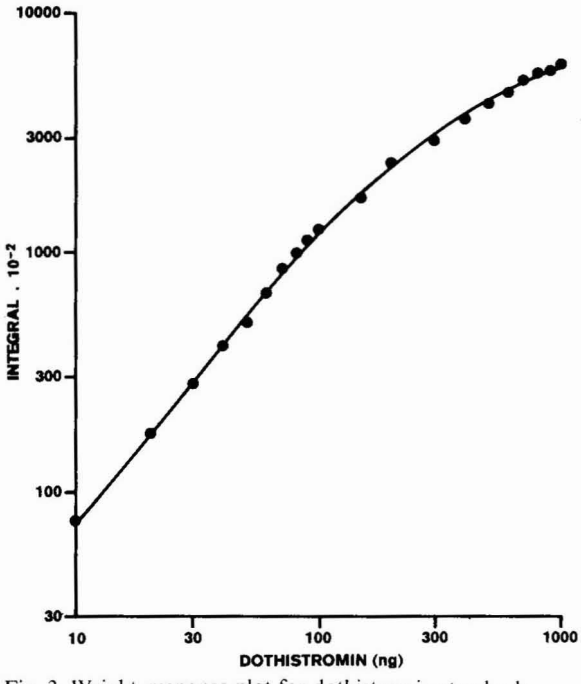

Fig. 3. Weight–response plot for dothistromin standards.

reversed-phase system. Precision of the analysis was determined by chromatographing ten 100-ng samples of dothistromin, and analysing by fluorescence densitometry. The mean integral ± standard deviation was equal to 100 ± 10.6 ng, *i.e.*, a 10% error can be expected for the determination.

For determination of dothistromin in pine needle extracts, three standards (10, 50, 100 ng) and nine samples are applied to each TLC plate. Time for sample application, chromatography, and analysis is 2 h.

In addition to providing a simple, rapid analysis for small quantities of dothistromin extracted from pine needles, the reversed-phase TLC described may also be useful for examination of foliage polyphenols and phytoalexins where interference from chlorophylls may be a problem[4].

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Note

Isotachophoretic characterization of stinging insect venoms

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(Received December 15th, 1980)

Venoms of common stinging insects, such as bees, yellow jackets, wasps and hornets, are complex mixtures of amines, peptides and proteins (enzymes) with both pharmacological and allergenic activities[1,2]. Honey bee venom has been extensively studied, which has resulted in the detailed biochemical characterization of most of the venom components[1,3,4].

Vespid venoms, on the other hand, have been subjected to few biochemical investigations, although King *et al.*[2] described biochemical and immunological studies of vespid venom proteins.

The use of modern biochemical separation techniques has made it possible to isolate individual venom components, and methods utilized for the purification and characterization of venoms from stinging insects of the order Hymenoptera are based on gel chromatography, ion-exchange chromatography and electrophoresis[2–4].

Isotachophoresis has been applied to the separation and quantitation of low-molecular-weight components[5] and proteins[6,7]. The high resolving power, in conjunction with the requirement for a small amount of sample, makes this technique very promising for the simultaneous separation and characterization of components in the venoms of stinging insects. In this paper an isotachophoretic procedure is described for studying bee and vespid venoms.

MATERIALS AND METHODS

Honey bee venom obtained by electrical stimulation was purchased from Mr. C. Mraz, Middlebury, VT, U.S.A. The venoms of wasp, white-faced hornet, yellow hornet and yellow jacket were purchased as lyophilized material from Dr. A. W. Benton, Pennsylvania State University, Spring Mills, PA, U.S.A. The vespid venoms were extracted from venom sacs. Melittin and bee venom phospholipase A_2 were obtained from Sigma, St Louis, MO, U.S.A., and histamine from E. Merck, Darmstadt, G.F.R.

The venoms were dissolved separately in distilled water to a concentration of about 1 %. Melittin, histamine and phospholipase A_2 were dissolved in distilled water to a concentration of approximately 0.1 %.

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The isotachophoretic analyses were performed with an LKB 2127 Tachophor (LKB, Bromma, Sweden), equipped with a 23-cm capillary for screening purposes. A 43-cm capillary was used for the identification of the peaks. The apparatus was equipped with a UV and a conductivity detector.

The leading electrolyte was 5 m*M* potassium hydroxide solution, adjusted to pH 6.0 with cacodylic acid (Pfaltz and Bauer, Flushing, NY, U.S.A.). To the leading electrolyte was added 0.2% (w/v) of hydroxypropylmethylcellulose (HPMC, Methocel 90 HG, 15,000 cps; Dow Chem., Midland, MI, U.S.A.) to minimize electroendosmosis. The terminating electrolyte was 5 m*M* creatine adjusted to pH 4.4 with hydrochloric acid. The spacer solution was 1 μl of 5% Ampholine. Carrier ampholytes of pH 6–8 were obtained from LKB.

The analyses were run at a constant current of 200 μA initially, which was decreased to 50 μA before detection (isotachophoretic equilibrium). The total time of analysis was 15–20 min, depending on the length of the capillary used. Samples of 2–5 μl were injected by means of a Hamilton syringe. The separated sample zones were detected by UV (transmission at 280 nm) and conductimetric detection. The transmission at 280 nm was recorded at a chart speed of 3 cm/min. The capillary was thermostated at 15°C.

RESULTS AND DISCUSSION

Fig. 1 shows the isotachophoretic separation of honey bee and vespid venoms in the presence of spacer solution (used in order to space the components according to

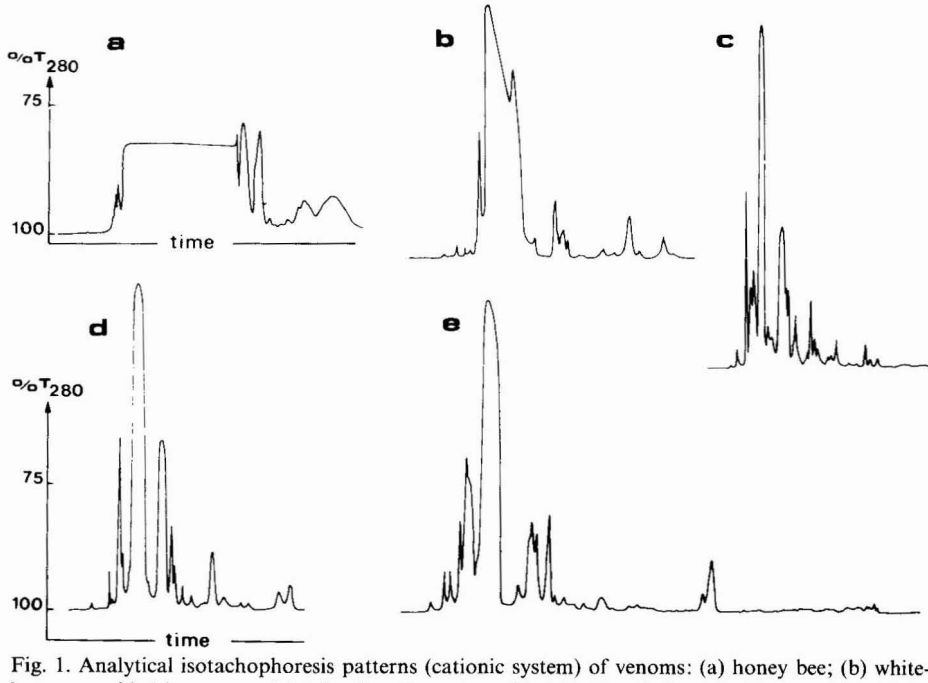

Fig. 1. Analytical isotachophoresis patterns (cationic system) of venoms: (a) honey bee; (b) white-faced hornet vespid; (c) wasp vespid; (d) yellow hornet vespid; (e) yellow jacket vespid. A 2-μl volume of each sample was injected with 1 μl of a 5% solution of spacer.

their mobilities), recorded with a UV detector (transmission at 280 nm). The UV profiles are characterized by a large number of absorbing zones (closely stacked peptide/protein patterns) for each venom species. On comparing the individual isotachophoretic patterns, it can be seen that honey bee venom (Fig. 1a) exhibits an isotachophoretic pattern with a major zone peak, which differs significantly from the vespid venoms. The long homogeneous zone in the honey bee venom profile indicates that one component is present in a large amount, as the zone width is directly proportional to the molar amount of an individual component. It is likely that this long zone is caused by melittin, as this peptide component constitutes about 50% of the dry weight of honey bee venom[1,2].

White-faced hornet, yellow hornet and yellow jacket venoms (Fig. 1b, d and e) exhibit similar isotachophoretic pattern, but wasp venom (Fig. 1c) shows a different profile, characterized by several well resolved zones. Inspection of the isotachophoretic profiles (fingerprints) of each venom species show, however, that it is possible to differentiate between the venoms investigated. Further, the results of several separate runs on each species of venom indicated very high reproducibility, which makes the isotachophoretic technique most suitable for routine identification purposes.

Fig. 2 illustrates the identification of the major component in honey bee venom. The isotachopherograms were recorded using both a UV and a conductimetric detector in the absence of spacer solution. The signal from both detectors demonstrates a high resolving power. Fig. 2a shows the separation of the complex peptide/protein mixture, and addition of melittin to the venom markedly increased the size of this homogeneous zone (Fig. 2b), which confirms that melittin is the major component.

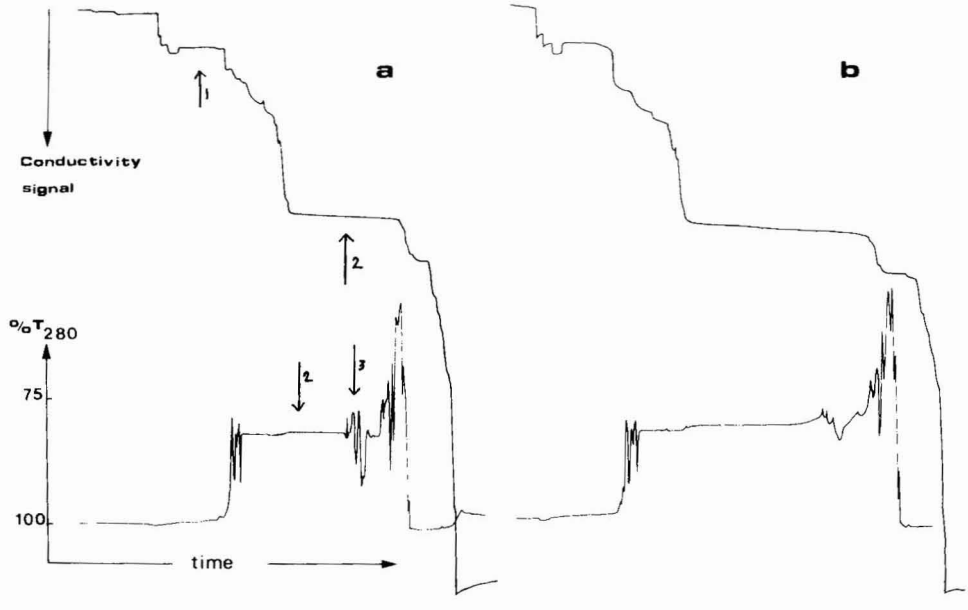

Fig. 2. Analytical isotachophoresis (cationic system) of (a) honey bee venom and (b) honey bee venom plus melittin. Arrows: 1 = histamine; 2 = melittin; 3 = phospholipase A_2. No spacer was used.

Further, the presence of histamine in honey bee venom has been verified. Because histamine does not absorb UV light, it must be detected with a conductivity detector, which is a general detector with high sensitivity (less than 50 pmol can be measured). Our studies indicate that histamine exhibits a high mobility in this electrolyte system and moves in front of melittin (Fig. 2a). Phospholipase A_2, on the other hand, showed a lower mobility than melittin (Fig. 2a). Further studies are in progress to quantify the individual components of hymenoptera venoms.

Analytical capillary isotachophoresis is easy to perform. Insect venom samples consisting of microgram concentrations in microlitre volumes can be analysed within 15–20 min. The results of these preliminary experiments suggest that this technique may provide a means for the rapid characterization of the venoms of stinging hymenoptera (fingerprints). Further, it might also give general information concerning the compositions of bee and vespid venoms.

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CHROM. 13,586

Letter to the Editor

Quantitative techniques in thin-layer chromatography

Sir,

In a recent detailed survey of the literature on quantitative thin-layer chromatography (TLC) we came across a paper in this journal[1] which, in our opinion, contains a misinterpretation of previous work by Bethke *et al.*[2] on the subject of the data pair technique and transferable calibration factors in TLC–densitometry[3]. Surprisingly, similar work has been also published in two other journals[4,5], but with differing conclusions regarding the relative merits of external *vs.* internal calibration.

Not considering the statistical aspects of the reported data, as the authors implicitly acknowledged the limitations associated with a lack of optimal working conditions and sufficient replicates[1], the following points should have been taken into account in writing or reviewing this paper.

(1) As stated by Grijalba *et al.*[1], in the data pair technique "a series of standard concentrations is applied on a plate. The procedure is repeated on the same plate with the same series of standards but with an equal amount of the unknown. The concentration of the unknown is then calculated from the intercepts of the curves with the concentration axis". However, the data pair technique, the only purpose of which is to compensate for fluctuating chromatographic conditions, requires the application of standards and unknowns in a paired fashion for each concentration and in such a way that the two spots of each pair are placed about one half-width apart[2]. In other words, the data pair method by itself is not a technique that quantitates TLC spots, but rather an ingenious approach to compensate for sources of variability across a TLC plate, thus eliminating systematic errors. Consequently, one should not confuse the simple standard additions method[6,7], which is what the authors are really using, with the data pair technique. The fact is that the latter could be used to improve the precision of the first or of any other quantitative methods for that matter.

(2) One of the methods also evaluated by Grijalba *et al.*[1] is that of transferable or external calibration, and in this regard it is not clear why, after determining the mean slope using five plates, *another* plate should be necessary for calculating the intercept on the ordinate and five more to calculate the precision and accuracy.

In fact, the ordinate intercept could be derived from any of the first five plates, which in any case would likewise have been incorrect as this value varies from plate to

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plate. What one needs to do instead is to "achieve a maximum of external information and a minimum of individual calibration", working with *one* calibration point on each plate[3].

Lacer S.A., Cerdeña 350, Barcelona-25 (Spain)

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Book Review

Gasatlas — Guide in gas analysis, Chrompack Nederland, Middelburg, The Netherlands, 1980, 39 pp., 1 table, 10 cards, price Dfl. 14.00.

The idea of this publication is good — by means of ingenious tables and 102 figures of gas chromatograms one can look up which gas mixtures have been separated and what results have been obtained.

The execution of this idea had me puzzled; then I remembered having read during a flight on a KLM plane an article in the KLM house journal explaining the "funny English" for which KLM hostesses are famous and which contributes to the fun of flying KLM.

However, in a scientific publication such involuntary humour is perhaps less welcome. On p. 1 one reads about "columns witching", in the cards there are compounds such as "aceton" and "nitrosubt. h. carb.", on p. 39 one is told to "Squeeze out of the paper strip the corresponding dots" and you are told "The number of the outhern spots...". On p. 2: "Very symmetrical molecules, which cannot enter into the micropores at no side...". To say nothing of a liberal sprinkling of spelling mistakes!

Summing up: A carefully edited and corrected edition will be very welcome and the present edition should be withdrawn.

Lausanne (Switzerland) M. GUDIENS

PUBLICATION SCHEDULE FOR 1981

Journal of Chromatography (incorporating *Chromatographic Reviews*) and *Journal of Chromatography, Biomedical Applications*

MONTH	N 1980	D 1980	J	F	M	A	M	J	J	A	S	O	N	D
Journal of Chromatography			203 204 205/1 205/2	206/1 206/2 206/3	207/1 207/2 207/3	208/1 208/2 209/1	209/2 209/ 210/1	210/2 210/3 211/1	The publication schedule for further issues will be published later.					
Chromatographic Reviews							220/1							
Biomedical Applications	221/1	221/2	222/1	222/2	222/3	223/1	223/2	224/1						

INFORMATION FOR AUTHORS

(Detailed *Instructions to Authors* were published in Vol. 193, No. 3, pp. 529–532. A free reprint can be obtained by application to the publisher)

Types of Contributions. The following types of papers are published in the *Journal of Chromatography* and the section on *Biomedical Applications:* Regular research papers (Full-length papers), Short communications and Notes. Short communications are preliminary announcements of important new developments and will, whenever possible, be published with maximum speed. Notes are usually descriptions of short investigations and reflect the same quality of research as Full-length papers, but should preferably not exceed four printed pages. For reviews, see page 2 of cover under Submission of Papers.

Submission. Every paper must be accompanied by a letter from the senior author, stating that he is submitting the paper for publication in the *Journal of Chromatography*. Please do not send a letter signed by the director of the institute or the professor unless he is one of the authors.

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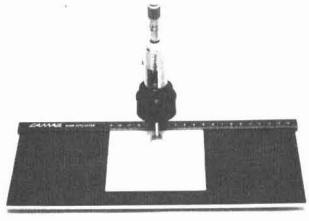

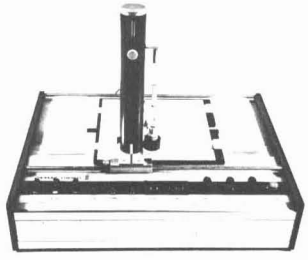

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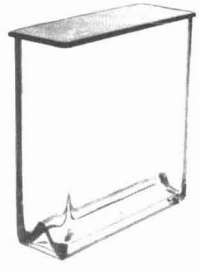

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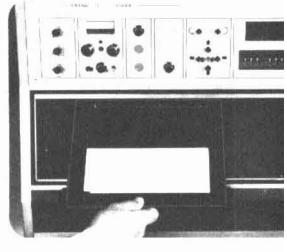

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